Sporulation and hybridisation of industrial strains of Saccharomyces cerevisiae and Saccharomyces carlsbergensis.

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SPORULATION AND HYBRIDISATION OF INDUSTRIAL STRAINS OF SACCHAROMYCES CEREVISIAE AND SACCHAROMYCES CARLSBERGENSIS

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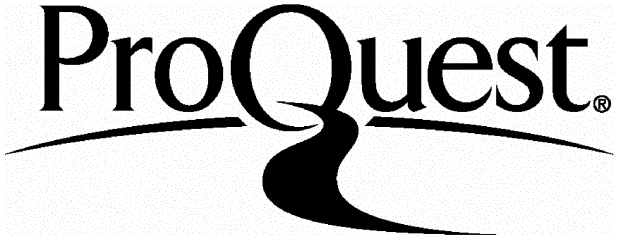

SUMMARY

A study was made of the sporulating behaviour of selected strains of Saccharomyces cerevisiae and Saccharomyces carlsbergensis and the fermentation properties of these 'parent' yeasts and the strains derived from them by hybridisation of mating isolates.

The brewing strains, in general, sporulated less readily than the baking, distilling and other strains. The 'lager' yeasts, strains of Sacch. carlsbergensis, had poorer overall sporulating ability than the 'ale' yeasts which were all strains of Sacch. cerevisiae. Most of the strains of Sacch. cerevisiae were shown to be heterozygous for mating type, and the proportions of strains of each mating type produced revealed that none of these brewing yeasts was simply diploid, but probably either triploid or aneuploid. Two of the strains of Sacch. carlsbergensis were apparently homozygous for mating type genes, and these strains were capable of hybridisation with strongly fertile mating strains derived from other yeasts.

The hybrid strains, which had been produced from compatible crosses between appropriate mating strains did not all have properties intermediate between those of their parent yeasts. Examination of the inheritance patterns among hybrid strains for their fermentation characteristics, namely flocculence, head-forming ability, duration of lag phase of growth, and attenuative ability, indicated that all of these properties are probably controlled by multiple gene systems, some of which may act in an additive way. In some of the crosses, the genes conferring flocculence exhibit classical dominance but, among hybrids derived from one brewing strain of Sacch. cerevisiae, non-flocculence was shown to be the dominant trait.

More detailed respirometric experiments, using selected hybrids as well as their parent yeasts and component mating strains, demonstrated that polymeric gene systems control the fermentation of the saccharides present in brewers' ale wort, and that the rate at which a yeast can ferment those sugars is related to its protein content, phase of growth and the composition of the wort.

Analytical results for beers produced by selected hybrid strains and control yeasts, in production-scale trials, revealed differences in total fusel oil contents, but these did not correlate with organoleptic evaluations.

CONTENTS

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INTRODUCTION

The alcoholic beverage, beer, has been produced for centuries and the techniques used have only changed slowly. Research into brewing technology with a view to improving the overall efficiency of the process is a fairly recent innovation.

Beer is produced by the alcoholic fermentation by yeast of an aqueous extract of malt, that has been flavoured with hops. This extract, known as brewers' malt wort, is partially clarified, diluted with water to the required specific gravity for the product, and then inoculated with a suitable yeast. The wort contains all of the necessary nutrients for yeast growth. The cells divide, and ferment the mono-, di-, and trisaccharides leading to the production of carbon dioxide and ethanol. The density of the wort falls and the final product at the specified gravity is beer. This is then filtered or treated with finings to remove yeast and other particulate matter, or 'lagered', depending on the type of fermentation carried out and beer required. Other materials may be added before packaging, such as hop extracts, colouring agents, syrups or head-retention aids.

Beers are classified into main groups according to the formulation of the wort (whether produced from 'ale' or 'lager' malt), the type of yeast used for fermentation, and the treatment of the product. Some yeasts settle to the base of the vessel during fermentation. These are used mainly to produce 'lager' beers and are normally strains of Saccharomyces carlsbergensis. The designation Saccharomyces uvarum is now recommended for this species by authorities on yeast classification (Lodder, 1970), but in the brewing industry the older name still predominates and therefore will be used throughout this thesis. Other

strains of yeast used in breweries rise to the surface of the fermenting medium in complexes of clumps of yeast cells known as flocs, which combined with bubbles of carbon dioxide form a yeast head. These 'top-fermenters' are usually strains of Saccharomyces cerevisiae and are utilised for ale production. This distinction is no longer as clear as in the past, for 'bottom-fermenting' strains of Sacch. cerevisiae are now used for ale fermentations in some types of fermenting vessels. The main taxonomic difference between these two yeast species (Rischbiet and Tollins, 1886; Berthelot, 1889) is the ability to ferment melibiose, for they are closely related to one another (Campbell, 1971). It is possible that they will eventually be interchangeable in ale and lager breweries. In addition strains of Sacch. carlsbergensis characteristically ferment better at lower temperatures. Hence they are used for lager fermentations which are conducted at temperatures of 10-16°C compared with temperatures of 17 to 20°C which are used for ale fermentations.

The physical behaviour of a yeast during fermentation is of great importance. As the specific gravity of the wort falls, the flocs of yeast cells may either rise to the surface of the fermenting wort or they may settle to the base of the vessel to form a sediment. Some strains combine the two types of behaviour and others remain in suspension throughout fermentation. Head-forming strains are used in brewery vessels where the yeast is harvested for re-inoculation by skimming, that is, removing the yeast from the surface of the fermenting wort. The second group, the bottom-fermenting strains, are suitable for use in

vessels where crops of yeast are collected from the base. A non-flocculent yeast (that is, one which remains in suspension) can only be utilised where total beer output is centrifuged for clarification.

The yeasts used at the present time in breweries are in many ways very satisfactory, but can be improved. Fermentation systems have been adapted and redesigned ever since the dawn of brewing, but during this century the rate of change has increased enormously. The 'ale' yeasts used in the past are not ideally suited to the modern types of fermentation vessels, which, for maximum fermentation efficiency must be filled above the level practicable if a top-fermenting strain is in use. These vessels are also frequently much larger than the ones they supersede, and have a different aspect ratio (the ratio of the height to the surface area). This can, in itself, promote a change in the physical behaviour of yeast cells, and may alter the metabolism of the strain and ultimately the flavour of the beer. Although harvesting procedures may exert a selective pressure on the yeast population so that mutants which are more suited to the new vessels predominate, this may be at the expense of desirable characteristics in the parent strain. For example, a new vessel may be designed for base-collection of yeast, which can in turn tend to select for more flocculent strains. This can lead to loss of attenuative ability and/or an alteration in the flavour of the beer produced. Such selective mutations may not therefore be able to provide an ideal yeast for a particular fermentation system.

The desirable properties of brewing yeasts are several. Firstly, the yeast should be able to ferment the mono-, di- and trisaccharides present in wort rapidly. The factors controlling the rate at which a

yeast strain will ferment wort saccharides are, firstly, the rates at which the sugars can gain access to the inside of the yeast cell, and secondly the rate of fermentation of these compounds by the intracellular or internal membrane-bound enzymes. The monosaccharide present in brewers' malt wort in the highest concentration, namely glucose, is transported into cells of Sacch. cerevisiae by a facilitated diffusion mechanism (Kotyk, 1961), is phosphorylated and metabolised largely by the Embden-Meyerhof glycolytic pathway. The pyruvate produced may then be used aerobically, when oxygen is available, by the tricarboxylic acid cycle to provide the cell with energy and also the precursors of nucleic acids and proteins, necessary for cell growth and maintenance. This method for producing the energy the cell needs would be predominant early in fermentation when oxygen is plentiful. Oxygen levels in worts vary from 2.8 ml/litre to 14.2 ml/litre depending on whether the wort is cooled in closed or open vessels and on the temperature of measurement (MacWilliam, 1968). As the oxygen supply becomes depleted, virtually complete after 90 min in a brewery fermentation (Pinnegar, 1965), the yeast cells then gradually change over to anaerobic mechanisms of obtaining energy, mainly by converting the pyruvate to acetaldehyde, which is in turn reduced to ethanol.

This anaerobic conversion of glucose to ethanol, an incompletely degraded end product, is not the most efficient means for a yeast to obtain energy. The change in free energy of the overall equation for alcoholic fermentation ($C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2$) is approximately -56 kcal per mole of glucose catabolised. The figure for the complete combustion of glucose is -686 kcal per mole, and therefore it is evident

that fermentation is a less economic means of obtaining energy (producing free energy and energy-conserving compounds such as ATP) than respiration. Hence, under anaerobic conditions, the yeast must ferment more sugar than when oxygen is freely available, in order to yield the required energy to live.

Thus, in brewery fermentations a balance is achieved between the energy-yielding pathways available to the yeast, dependent on fermentation conditions. Glucose is normally broken down, mainly by the Embden-Meyerhof pathway, to pyruvate, which, under aerobic conditions, is converted to acetyl CoA. This compound is the starting material for the citric acid cycle, a pathway which provides reducing power and precursors for the anabolic processes necessary to synthesise new cell components. As oxygen becomes limiting, the yeast cells change their metabolism, and ferment the pyruvate formed anaerobically, decarboxylating it to acetaldehyde. Most of the acetaldehyde formed is reduced to ethanol and carbon dioxide but, under certain conditions this conversion may be blocked, causing a build-up of dihydroxyacetone phosphate, which is, in turn, reduced to glycerol. The other monosaccharide present in brewers' malt wort, fructose, is taken up and metabolised in a similar manner to glucose, but this sugar is directly phosphorylated by hexokinase on entering the hexose monophosphate pathway, and the enzyme glucose phosphate isomerase is obviously not utilised.

In order to attenuate wort sufficiently, the yeast must be able to metabolise as much as possible of its fermentable carbohydrates. The main carbohydrates in all-malt ale worts of specific gravity 1.040 are glucose, fructose, sucrose, maltose and maltotriose at concentrations of

1.0, 0.3 to 0.9, 0.5 to 0.6, 3.9, and 1.1 to 1.3 g per 100 ml of wort respectively (MacWilliam, 1968). In lager worts of similar starting gravities, the concentrations are similar, namely 0.7 to 1.3 g of glucose per 100 ml, 0.1 to 0.3 g fructose per 100 ml, and the higher saccharides sucrose, maltose and maltotriose at concentrations of 0.1 to 0.38, 5.0 and 1.2 to 1.5 g per 100 ml. Non-fermentable carbohydrates form a further 5 g per 100 ml. The most important fermentable saccharides present in wort, therefore, apart from glucose and fructose are sucrose, maltose and maltotriose which are initially converted into their component monosaccharides, glucose and fructose from sucrose, and glucose molecules only from maltose and maltotriose. The monosaccharides then either undergo glycolysis by the Embden-Meyerhof pathway to give ethanol and carbon dioxide, or are catabolised via the tricarboxylic acid cycle after conversion to pyruvate, as described above.

Sucrose is broken down by a yeast-wall enzyme, β-D-fructofuranoside fructolyase, and the glucose and fructose are then transferred through the cell wall and the membrane by facilitated diffusion. The disaccharide maltose, the predominant saccharide in malt wort, and the trisaccharide maltotriose are both transported through the cell wall and membrane by the action of permeases, before making themselves available to the enzyme α-D-glucoside glucohydrolase. The actual position of this enzyme, otherwise known as 'maltase', in the cell is uncertain, for it has been suggested (Suomalainen, 1948) that bakers' yeast contains two different α-glucosidases, one internal, and the other, active in acid media, located on the surface. However, in brewers' yeasts which lack α-D-glucoside glucohydrolase activity in such media, the enzyme seems to

be located in the cell wall. Brewers' yeasts have been shown to be able to decompose cellobiose, but very slowly (Weidenhagen, 1930).

Fermentation of maltose, the major carbohydrate component of malt wort, is reported to be controlled by polymeric genes in Saccharomyces species. Altogether up to six genes may be responsible for the production of maltase, otherwise known as α-D-glucoside glucohydrolase(3.2.1.20) (Winge and Roberts, 1958; Oeser and Windisch, 1964). This enzyme is able to split α-glucosidic linkages in maltose and maltotriose to produce separate molecules of glucose. However the ability of a yeast strain to ferment these two saccharides also depends on the presence of the appropriate permease system which allows transport of the molecules across the cell membrane to make them available to the α-D-glucoside glucohydrolase. An additional gene has been demonstrated which determines the production of maltotriose permease (Oeser and Windisch, 1964). It is not yet known whether this gene (M 7) is identical with one of the two genes reported to be present in Sacch. diastaticus by Lindegren and Lindegren (1956).

This polymeric system of genes responsible for fermentation of these two sugars means that the wide range of fermentation efficiencies obtained for the metabolism of maltose and maltotriose can be related to the gene dosage for the production of maltase, on condition that the yeast cells have the necessary effective permease systems. Therefore the more genes a yeast has which determine the production of this enzyme, the faster it will ferment the di- and tri-saccharides, for the induced level of α-D-glucoside glucohydrolase increases arithmetically with increased numbers of these genes (Halvorson et al, 1963). This gene-dosage effect can

therefore mean that a fast-fermenting hybrid strain may be produced from two less efficient component mating strains (Fowell, 1958). In addition a further allele has been found (MAL 4) which permits constitutive synthesis of maltase (Zimmermann, et al, 1973). In the presence of this gene, a yeast can ferment sucrose in the absence of genes determining the production of invertase, β-D-fructofuranoside fructolyase (3.2.1.26) although the mechanism whereby this occurs has not been explained (Khan et al, 1973). It is suggested that, if sucrose can penetrate the cell membrane, a high level of maltase should be able to hydrolyse it, although it must be emphasised that the linkages in the two disaccharides differ. These workers (Khan et al, 1973) identified in one yeast stock a recessive gene which interfered with sucrose fermentation in the strain probably by limiting the penetration of sucrose.

A polymeric system, similar to that responsible for the fermentation of maltose is also involved in determining the production of the cell wall-bound enzyme which hydrolyses the disaccharide sucrose. The enzyme, β-D-fructofuranoside fructolyase (3.2.1.2.26, otherwise known as invertase) is specified by up to five genes (Ottolenghi, 1971) and they also are additive in effect. The other major sugars in wort, glucose and fructose, are transported into the cell by a facilitated diffusion mechanism (Kotyk, 1961) and fermented by the normal glycolytic pathway, pyruvate formed being converted into acetaldehyde, which is then reduced to ethanol, as already mentioned.

A short lag phase and good growth rate are also desirable properties in a brewery yeast strain. Overall fermentation rate is related to the

extent of growth and yield of a yeast (Brown and Kirsop, 1972). The growth rate and duration of the lag phase of a strain, like the fermentation rate, are controlled by the rate of uptake of nutrients, enzyme synthesis, and the efficiency with which these nutrients can be converted into new cell material and energy. In a brewery fermentation, an increase in cell numbers occurs until one nutrient, probably molecular oxygen, becomes limiting. Therefore maximum yeast counts are reached fairly early in the brewery fermentation period, and normally within the first 30 hours. Some mention has already been made of the tricarboxylic acid cycle which is the pathway for the catabolism of pyruvate under aerobic conditions. This cycle, as explained before, yields precursors of the components needed for new cell material, proteins, nucleic acids and lipids, although some of these compounds or their precursors can be obtained directly from the medium. Wort contains quantities of all these materials, and is particularly rich in amino acids, peptides and proteins, which combine to account for up to 40 mg of α-amino nitrogen per 100 ml (MacWilliam, 1968). However not all of these can be taken up or metabolised by the yeast cells, for example, proline, which comprises up to 33 per cent of the total amino-acid content. Up to six per cent of the total nitrogen in wort is present as purines and pyrimidines (Laufer et al, 1951), of which a proportion is unbound (Harris and Parsons, 1957) and therefore readily available to the yeasts, like some of the fatty acids and lipids which total five to seven mg per 100 ml.

Pyruvate, which as has been mentioned, is necessary to produce all compounds which cannot be obtained directly from the medium, is prepared from the metabolisable sugars which are in abundance in wort, and its

production is therefore controlled partly by some of the enzymes involved in anaerobic glycolysis and also by the enzymes α-D-glucoside glucohydrolase and β-D-fructofuranoside fructolyase. Many yeasts show marked inhibition of respiratory capability when abundant glucose is available in the medium (Ephrussi et al, 1956). This may be due to 'catabolite repression', to the breakdown of mitochondrial structures under these conditions, or a combination of other factors, but which of these is responsible is not yet clear. The extent to which the fermentative pathways from pyruvate to ethanol are used can be affected by the presence of the respiratory enzymes (Tustanoff and Bartley, 1964) and/or repression of pyruvate decarboxylase (Suomalainen and Oura, 1959), the enzyme responsible for the breakdown of pyruvate into acetaldehyde. Further regulation of metabolism is brought about by a decrease in the rate of glucose utilization in aerobic conditions, compared with the rate under anaerobic conditions, known as the Pasteur effect. This is brought about by feedback inhibition at various levels in the main series of pathways of glucose degradation, and controls the amount of material diverted into the manufacture of cell components.

In brewery fermentations, a balance is required between the aerobic and fermentative pathways for, although a high yeast concentration is desirable, an excessive increase in cell mass is not required, because the sugars are then being channelled into the production of new cell matter, instead of being converted into ethanol. In addition, yeasts which grow too rapidly produce by-products which can affect flavour (Coote et al, 1973). Larger inocula or higher 'pitching rates' are frequently used in breweries where the strain has a prolonged lag phase, but this should be

unnecessary with a more efficient yeast. In addition, an initially rapid rate of growth of the yeast population minimizes the risk of infection from bacteria or wild yeasts because competition for nutrients and rapid fall in pH value prevent the contaminants becoming established.

In certain fermentation systems, particularly the continuous types (Hough and Rudin, 1959; Mukhopadhyay and Ghose, 1962) a fast growth rate and high specific growth rate constant are very important. A constant population of cells must be maintained in the fermenting vessel or vessels, and therefore the more rapid the growth rate of the strain, the faster the wort can be introduced into the system, thereby increasing the dilution rate, and the rate of beer production.

The third property of yeast cells which also determines their suitability for beer production is their physical behaviour during fermentation, which must be suited to the harvesting and fermentation methods in use. As already mentioned, head-forming yeasts which often grow in chains of cells, are suited to the skimming methods used for collection of crop in 'Burton Union' and 'Yorkshire Stone Square' vessels. A degree of sedimentation of flocs is usually also required in these yeasts, so that the final beer does not require centrifugation, but can be clarified by the addition of flocculating agents, such as 'finings'. Flocculent non-head-forming strains of yeast are recommended for use in Nathan batch fermenters (Nathan, 1908, Shardlow, 1971), and Universal Tanks (Knudsen and Vacano, 1972) where the yeast is run off from the basal cone, and also for the continuous systems such as cylindrical fermenters (Portno, 1969), gradient tubes (Portno, 1968) or the chemostat vessels with an

overflow transfer (Coutts, 1961, 1966), where the yeast is collected after sedimentation. In breweries where the beer is centrifuged, a non-flocculent strain of yeast may be implemented.

The phenomenon of flocculence is not yet completely explicable. The reactions that lead to flocculation appear to take place only at the surface of the cell wall, for isolated cell walls have been shown to exhibit the same flocculating behaviour as the intact cells (Masschelein, 1957, Masschelein and Devreux, 1957). It has proved difficult to analyse the effect of external influences on cells prior to flocculation because of the variable composition of wort. Koch (1928) demonstrated that the pH of initiation of flocculence for a yeast strain is not constant. Yeast cells can flocculate in a synthetic medium containing only glucose and an ammonium salt, showing that the more complex constituents of wort are not agents initiating flocculence (Eddy, 1955a). Calcium ions have been observed to enhance flocculation, especially at pH values between three and four (Mill, 1964). Other ions, acids and ethanol can influence the flocculating properties of different yeast strains (Eddy, 1955b). In addition it has been observed that factors which stimulate yeast growth, such as sugars (Eddy, 1955b; Nielson, 1937), will inhibit flocculation, and that flocculent yeasts will not agglutinate until they reach the stationary phase of growth (Masschelein and Jeunehomme-Ramos, 1959). These workers later also showed (Masschelein *et al*, 1963) that the mannan content of the yeast cells rose to a maximum and fell again during the fermentation period and suggested that this compound masked the agent which caused flocculation. Recent work (Deutch and Parry, 1974) has shown that yeasts

from stationary-phase cultures are more resistant to the action of glucanase than log-phase cells. This suggested that, in progressing from the logarithmic to the stationary phase of growth, changes occur in the wall composition. Deutch and Parry (1974) obtained evidence in favour of this view, for if yeast cultures were treated with cycloheximide or 5-fluorouracil in the log phase of growth, they could readily be converted into sphaeroplasts in the stationary phase. This demonstrates that cell-wall structure 'hardens', that is, becomes more resistant to enzymatic attack as the cells age.

The effect of wort proteins on yeast flocculation (Ranken, 1927, 1928; Ito, 1967a) initiated interest in the colloidal aspects of this phenomenon. A number of workers have attempted to investigate the behaviour of compounds in the cell wall of yeast, using viscosity experiments at different pH values (Lüers and Heusz, 1921) or by micro-electrophoresis (Geys, 1922; Silbereisen, 1938; Hennig and Ay, 1938), but none of these experiments produced conclusive results relating changes in charge to the onset of flocculation. Winslow and Fleeson (1926) demonstrated that, in water, yeast cells have a negative charge, but could not neutralise this charge in their experiments. However Moldawskaja (1933) showed in cathaphoretic experiments that reversal of charge could be effected with ammonium chloride solution. Other experiments hinged on the suggestion that yeast cells may secrete a gum-like substance which could cause cells to aggregate and flocculate (Stockhausen and Silbereisen, 1933, 1935), but this theory was not proved.

More recent experiments have shown that, early in fermentation, yeast cells have a high negative charge density which alters during wort fermen-

tation (Jansen and Mendlik, 1951). In addition the electrophoretic mobility of cells has been shown to decrease during the fermentation period (Eddy and Rudin, 1958). A relationship has since been demonstrated between the negative charge density on the surface of a yeast cell and the phosphate groupings on the cell wall (Ito, 1967b). Some phosphate-containing compounds are related to energy metabolism and their amounts dynamically change as the cells age, and as their enrivonment alters. Lyons and Hough (1971) have also shown that the capacity of cells to flocculate by cross-bridging is proportional to the number of phosphate groups present in the outer layer of the mannan-protein. They suggest that the level of phosphate is sufficient only in flocculent cells for binding to occur between adjacent cells in the presence of divalent cations. In addition helicase, a mixture of enzymes derived from the gut of the Roman snail, Helix pomatia, has been used to induce flocculation in stationary phase cultures of flocculent strains of Saccharomyces cerevisiae, but not in powdery strains (Geilenkotten and Nyns, 1970). These workers suggested that a cell-wall enzyme might be involved in the initiation of yeast flocculence.

The phenomenon of flocculence is therefore still poorly understood, and methods of measuring it are numerous. Most of these methods depend on the sedimenting behaviour of flocs of yeast (Burns, 1937; Helm et al, 1953; Nielson, 1937; Jansen and Mendlik, 1951; Chester, 1963) but others utilise deflocculating agents (Eddy, 1955b) or microscopic examination of flocs (Gilliland, 1957).

The fourth important consideration when choosing a suitable brewers' yeast is the flavour of the beer that the strain produces when it has

fermented brewers' wort. The yeast cells must be able to form during fermentation a balanced level of minor products, by-products of metabolism, such as acids, ketones, higher alcohols and esters, which in addition to certain components of hop acids, ethanol, unfermented sugars and nitrogenous compounds help to make a palatable beer. These compounds arise from carbohydrate, lipid and nitrogen metabolism and are reported to confer various flavour characteristics. For example, among the aromatic 'higher' alcohols, phenyl ethyl alcohol can produce a perfumed aroma, tryptophol and tyrosol a bitter flavour, and the aliphatic fusel alcohols, amyl alcohols, 'fruity' aromas (Voerkelius, 1966). The formation of fusel alcohols is closely linked to nitrogen metabolism, high concentrations being produced under conditions of nitrogen limitation (Ayrapää, 1973), whereas the formation of esters is connected with lipid metabolism. A high content of esters is also undesirable, for it gives beer a harsh flavour, and acetaldehyde and diacetyl can give unpleasant characteristics to the product. In addition, sulphur compounds, such as thiols, mercaptans and dimethyl sulphide even at low concentrations can affect beer flavour.

There is evidence that the production of these compounds can be influenced by growth rate (Coote et al, 1973), concentrations of dissolved oxygen in the wort (Coutts, 1966; Portno, 1968), flocculating behaviour of yeasts (Thorn, 1971), or by the proportions of sugars and low molecular-weight nitrogenous materials in the wort (Voerkelius, 1966; Engan, 1970) or fermentation temperature (Kamiyama and Nakagawa, 1966). It is conceivable that in the future breweries will be able to control beer flavour by maintenance of suitable conditions.

Fifthly, the yeast strain must remain genetically stable, particularly where it is harvested for re-inoculation of a subsequent fermentation. In continuous fermentations, an 'unstable' strain will be constantly throwing off mutant types which may take over the fermentation, where conditions or harvesting methods favour their survival. Many types of mutation arise spontaneously in a yeast population. Yeast 'degeneration' or 'weakening' has been noticed in breweries for many years (Devreux, 1962). Incomplete attenuation of wort, that is, an insufficient drop in specific gravity may result, producing what are known as 'hanging fermentations'. This may be caused by the natural selection by harvesting or outgrowth of mutant strains which are either more flocculent or which have decreased attenuative efficiency due to decreased activity of transport proteins and/or enzymes involved in glycolysis of the wort saccharides. Alternatively, these poorly attenuated fermentations may occur because the wort is deficient in essential yeast nutrients, such as vitamins. Mutation to a less flocculent strain is common, particularly in continuous fermentation systems (Thorne, 1962; Thorne and Nǿhr, 1963), in fact the frequency of mutation of the genes for flocculence has been reported to be as high as 5.3 per cent in cultures derived from spores (Thorne, 1952). During vegetative growth, the mutability of single flocculence genes has been estimated at one in every 5,000 per generation time (Thorne, 1952). Obviously, in a yeast which has, say, three pairs of 'dominant' genes for flocculence, the mutation to a non-flocculent strain will be far less likely, that is, one cell per 15×10^{21} per generation time. Such

non-flocculent strains would probably have a faster growth rate than the parent type, and could take over, causing problems in beer clarification.

In lager fermentations, mutation to 'petites', respiratory-deficient strains (Bulder, 1963) occurs relatively frequently. These mutants, which are very flocculent however, rarely cause problems because of their slow growth rates, unless they are preferentially selected by harvesting the earliest-formed bottom crop.

Another important characteristic of a yeast is its ability to produce, in a brewery fermentation, a sufficiently large crop which can be readily harvested for inoculation into subsequent fermentations. This depends on the overall growth rate and fermentation efficiency of the yeast, and its flocculation behaviour. In addition, yeast strains vary in their ability to remove hop-bittering substances (Meilgaard et al, 1955), from the fermenting wort, and it is economically desirable to choose a strain which removes as little as possible.

All of these characteristics of brewing yeasts are governed by the genetic make-up of the yeast as well as environmental conditions. Mutations in the base sequence of the deoxyribonucleic acid in the cell will change the behaviour of a yeast strain. Such alterations are already known to be possible using mutagenic agents such as radiation, chemicals like ethyl methyl sulphonate and bromo-uracil, or dyes. Reports of transformation (Oppenoorth, 1959) of DNA between yeast cells, and zymophage (Lhaos, 1972) and killer factors (Woods and Bevan, 1968; Bussey, 1972) suggest that, in the future, it may be possible to introduce DNA

into strains by mechanisms used commonly in 'genetic engineering' of bacterial cells. It has also been suggested that other types of episomal DNA are present in yeast cells, and that flocculence genes have a high rate of mutation (Thorne, 1952) because they are extranuclear. Mitotic recombination, a further means of altering the base sequence in the nucleic acids, has also been demonstrated to occur spontaneously in strains of Sacch. cerevisiae, and can be induced (Zimmermann et al, 1966) using nitrous acid, diethyl sulphate and certain nitrosamides. Finally, alternation of generations, that is, of haploid and diploid phases, which can occur under certain conditions allows the conjugation of genetic material from different strains of Saccharomyces to be contrived. Such a technique, known as 'hybridisation', can be used to introduce new characteristics into existing strains of yeast.

Considering these alternatives, only one method, namely hybridisation seems to be suitable for producing yeasts with improved fermentation efficiency. Although mutagenesis has proved useful industrially for increasing yields of by-products of metabolism such as penicillin (Gattani, 1952), it is doubtful whether overall metabolism could be improved in this way. Transformation of DNA, common between related strains of bacteria, has only been reported to occur in yeasts by one worker, (Oppenoorth, 1959, 1961), who 'fed' a yeast strain with DNA extracted from another. However, reports on the use of this technique for the production of new yeast strains have not yet appeared. This would indicate that it is either a very complex method, or that there has been no success. In addition, it would be impossible to ascertain whether transfer of DNA

had occurred between industrial strains of brewing yeasts, for these strains have no easily-monitored genetic markers. Episomal nucleic acids have only recently been discovered (Lindegren and Bang, 1961; Lhaos, 1972; Woods and Bevan, 1968) and there have been no reports of attempts to use such factors for 'transduction' of genetic material. Hybridisation is a proven technique, used to prepare new strains of bakers' yeast (Burrows and Fowell, 1961a, 1961b, 1969) distillers' and brewers' yeasts (Gilliland, 1951) and strains which produce increased alcohol yields from molasses (Kosikov, 1963).

Hybridisation of yeasts depends on the alternation of generations in yeast species, the presence of which was established by Winge (1935). Six or more types of life cycle have been described in yeast-like fungi with differing lengths of haplo- and diplophases (Phaff and Mrak, 1948; Raper, 1954). The life cycle of yeasts of the species Sacch. cerevisiae and Sacch. carlsbergensis is predominantly diploid, with only a short haplophase, consisting of the spores or haploid cultures prior to mating. Typically the diploid vegetative cells develop into asci containing up to four spores, originally thought to be formed by a meiotic division followed by a mitotic division. Although cytological evidence for these divisions has, until recently, proved difficult to obtain with the electron microscope (Hashimoto, et al, 1960) dense bodies staining with nucleoprotein dyes have been demonstrated in some Saccharomyces species (McClary et al, 1957a and b). However more recent work, using electron microscopy (Moens, 1971; Moens and Rapport, 1971) has shown that the divisions

involved are both meiotic, and that a four-lobed nucleus is produced prior to the formation of separate spore bodies.

Demonstration of separate chromosomes has also proved difficult in yeasts, using staining or microscopic techniques, but eighteen double sets of chromosomes have been shown during a possible metaphase in one strain of Sacch. cerevisiae (Tamaki, 1965). Genetic analysis has revealed that another strain of a Saccharomyces species has at least fourteen linkage groups (Mortimer and Hawthorne, 1966, 1973) and even more groupings have been demonstrated in one strain of Sacch. cerevisiae (Hawthorne and Mortimer, 1968). Of the genes involved in the fermentation of brewers' malt wort only a few have been mapped, namely, three of the genes responsible for the production of α-D-glucoside glucohydrolase, MA 1, MA 2 and MA 4, and two determining the production of β-D-fructofuranoside fructolyase, SU 1 and SU 2 (Mortimer and Hawthorne, 1966). These are some of the genes which determine whether or not a strain can ferment maltose and sucrose. The majority of the genes that have been mapped in this strain of Saccharomyces cerevisiae are requirements for particular amino acids or resistance to antibiotics, and therefore difficult to correlate with brewery fermentation efficiency. The 'flocculence' genes have not, as yet, been mapped.

Yeasts of the genera Saccharomyces, Saccharomycodes, Hansenula, Pichia, Endomycopsis and Hanseniaspora generally produce a maximum of four spores in an ascus. Other yeasts, such as strains of Nematospora and Coccidiascus and also Schizosaccharomyces octosporus, sporulate to give asci containing up to eight spores. More than eight spores may be produced by strains of Saccharomyces (Winge and Roberts, 1950; Lindegren

and Lindegren, 1953; Santa María, 1957), Lipomyces species (Lodder and Kreger van Rij, 1952) and Schizosaccharomyces pombe (Gutz, 1967). Not all asci from one yeast strain contain the same number of spores, because one or more of the daughter nuclei resulting from the meiotic divisions may fail to be incorporated into a spore (Nagel, 1946; Pontefract and Miller, 1962). Brewing yeasts, strains of Sacch. cerevisiae and Sacch. carlsbergensis rarely produce four-spored asci (Fowell, 1969), two- and three-spored asci being normal for the former, and one- and two-spored ones for strains of the latter species. Indeed some yeast strains have entirely lost the ability to sporulate, probably because of abnormal ploidy or homothallism (Winge and Roberts, 1954; Emeis, 1958) and the haplophase of the life cycle is eliminated.

Conditions for induction of sporulation have been studied extensively. Originally starvation was thought to be the cause (de Seynes, 1868; Hansen, 1902). However certain nutrients are necessary for optimal sporulation and environmental conditions are very important. No single medium is suited to all yeast strains. Gypsum blocks (Phaff and Mrak, 1949), vegetable slices (Bedford, 1941; Phaff and Mrak, 1949) and vegetable extracts (Wickerham et al, 1946) have all been used in the past to induce sporulation.

However, more recent studies have revealed that the presporulation phase is important and actively growing cells give optimal sporulation when transferred to a suitable sporulation medium (Hansen, 1883; Fowell and Moorse, 1960). Cell population density (Fowell, 1967; Kirsop, 1957) may also be critical and temperature (Stantial, 1935; Adams and Miller, 1954), carbon and nitrogen substrates, pH value and organic ions of the medium play a vital role (Fowell, 1969).

After sporulation, the spores need to be isolated, ready for hybridisation. Methods of rupturing the asci are numerous, the most common being enzymic, using extracts of bacteria (Wright and Lederberg, 1957; Magni and von Borstel, 1962), or snail gut juice (helicase; Johnston and Mortimer, 1959). Mechanical techniques have also been implemented (Emeis, 1958; Emeis and Gutz, 1958; Windisch, 1961), but damage to spores is then unavoidable. Both of these methods require supplementary treatment to separate the spores which adhere together after removal of the ascus wall. For rupture of asci where genetic analysis is to follow, micromanipulation is used to separate asci, and dissect out the spores (Fowell, 1969).

Some strains of brewers' yeast sporulate poorly and/or produce spores of low viability or fertility (Fowell, 1969), and methods of concentrating the spores in the mixture with asci, and vegetative cells may be necessary. Selective heat treatment (Wickerham and Burton, 1954) is said to eliminate a proportion of the vegetative cells, because they are slightly more sensitive to higher temperatures than are spores. Other techniques, used for concentration, are based on the difference between surface charge characteristics of vegetative and spore cells, and involve oil flotation (Gutz, 1958) or electrophoresis (Resnick et al, 1967).

When these separation methods are not used, the cells may disperse when the ascus wall is ruptured. However, in some strains of Sacch. cerevisiae and Sacch. carlsbergensis, the spores self-diploidise, (Winge and Laustsen, 1940; Fowell, 1969), that is, become diploids homozygous for mating type by doubling their chromosome number. Hence the haplophase of such yeasts is reduced to a minimum. In other strains of Sacch. cerevisiae, there is a 2:2 segregation of diploid and haploid spore

cultures (Fowell, 1958). Diploid spores may also be produced due to the inclusion of two daughter nuclei in one spore (Winge and Roberts, 1950) or by polyploid yeasts such as those used in breweries (Emeis, 1958).

The next stage after sporulation and separation of haploids is the mating procedure. Matings may be carried out in one of three ways. Originally spores were paired in droplets of wort (Winge and Laustsen, 1938) which eliminates the possibility of self-diploidisation. However, the low rate of fusion (Roberts, 1950) and the fact that spores can only be used once makes this technique unsuitable for industrial breeding projects. The second method entails growing 'haploid' cultures from the isolated spores, isolating single cells from these by micromanipulation and mating these, watching for conjugation under the microscope. However, nuclear fusion does not occur in some instances (Fowell, 1969) and haploid cells may be budded off the apparent zygote. The third technique commonly used in the production of industrial hybrids is known as mass mating (Lindegren and Lindegren, 1943à) and entails mixing large numbers of cells of determined opposite mating type in a nutrient medium and incubating. Hybrid cells can be isolated from this mixture by either micromanipulation or outgrowing the slower haploids by successive subcultures. This last method has the advantage that it does not involve micromanipulation, and consequently larger numbers of hybrids can be obtained.

In polyploid yeasts, such as brewing strains of Saccharomyces cerevisiae and Saccharomyces carlsbergensis (Emeis, 1958), a high percentage of sterile spores are produced (Fowell, 1969). In addition those yeasts which can mate with the standard strains used for mating type determinations are not always compatible with other 'mating strains' derived from

brewing yeasts. Theoretical ratios of the numbers of a to α to sterile spore cultures for yeasts of different ploidy and constitution have been derived (Emeis, 1958). For example, a triploid yeast which has the complement a a α gives a segregation of spore cultures a:α:sterile of 3:1:2, or one which is composed of a α α gives a ratio of 1:3:2. Therefore for both these types of yeasts, one third of their spore products will be sterile. Where tetraploid yeasts are concerned, the constitutions a a a α, a a α α and a α α α would, in theory, give rise respectively to spore cultures in the ratio of a:α:sterile of 1:0:1, 1:1:4 and 0:1:1, that is either two thirds or half of the spores will be sterile. The proportion of sterile products rises as the ploidy number increases. Thus in pentaploid yeasts of mating type constitution a a a α α a ratio of 4:1:15 will be obtained and of the complement a a α α α, 1:4:15, three quarters of the total. Hybridisation of known haploids, however, produces diploid hybrids which, theoretically, should give low percentages of sterile mating strains. This technique can be used to improve the overall fertility and viability of spore cultures to be used in breeding programmes.

The inclusion of favourable fermentation characteristics in a particular yeast hybrid strain depends on the way that these factors segregate during sporulation of the parent strains and the influence that the genes of one mating strain have on those of its partner during and after hybridisation. The most favourable hybrids are not always produced from the best haploids or parent yeast (Fowell, 1958), due to the additive or subtractive effect of some genes, particularly those concerned with sugar

fermentation (Oeser and Windisch, 1964). Some characteristics, such as the rate of utilisation of these sugars in wort, may be vastly improved in hybrids when compared with their parental types (Fowell, 1958; Clayton, et al, 1972). However other characteristics such as flocculence may be intermediate between those of the parent types (Gilliland, 1951; Kirsop, 1970). Inheritance of flocculating ability has been studied by several workers, and the variable conclusions reached reveal the heterogeneous nature of the phenomenon termed 'flocculence' in yeasts. A single dominant factor was proposed as the determinant of flocculence in one strain (Gilliland, 1951) but genetical investigations of another group of brewing yeasts have disclosed the presence of three polymeric genes determining this property (Thorne, 1951a, 1952). Flocculence was again concluded to be a dominant trait because the presence of a single dominant gene conferred flocculating ability on its hybrids. However, non-flocculent strains sometimes arose where they could not be expected from calculated ratios and this was explained by an excessively high mutation rate from the dominant to the recessive gene, or an inhibitor or repressor gene (Thorne, 1952). It may be that the three-gene polymeric system is not the whole explanation, for other work (Gilliland, 1951, 1955; Kirsop, 1970) has shown that flocculence is genetically controlled but in all cases it was not clear whether it or non-flocculence was the dominant trait.

The object of the work in the present study is to examine the sporulation characteristics of strains of Sacch. cerevisiae and Sacch. carlsbergensis and to produce by hybridisation strains of yeast which, together with the parent strains, can be examined for inheritance of physical and biochemical factors.

MATERIALS AND METHODS

ORGANISMS

The yeast strains employed in this work are listed in Table 1. These strains and all hybrids and mating strains were maintained on MYGP agar slopes (Wickerham, 1951) at 4°C and subcultured every three to six months.

MEDIA

Malt Extract-Yeast Extract-Glucose-Peptone Medium (MYGP; Wickerham 1951)

	g/litre
Malt Extract (Oxoid)	3
Yeast Extract (Difco)	3
Glucose	10
Peptone (Difco)	5

The medium was dispensed in 10 ml portions in MacCartney bottles (30 ml capacity). To make MYGP solid medium, agar (15g/l; Oxoid No 3) was included; the medium was placed in a steamer to melt the agar and then dispensed in MacCartney bottles. Both MYGP broth and MYGP agar were autoclaved in 15 lb/in^2 for 15 min. The bottles containing melted agar were inclined at an angle of ca 30° after autoclaving to produce slopes of solidified medium.

TABLE 1

Strains of Saccharomyces cerevisiae and Saccharomyces carlsbergensis employed in the present study

Strain number	Origin or designation
Saccharomyces cerevisiae	
Y 1	Ale brewery strain
Y 7	Ale brewery strain
Y 8	Ale brewery strain
Y 9	Ale brewery strain
Y 10	Ale brewery strain
Y 11	Ale brewery strain
Y 26	Ale brewery strain
Y 32	Bakery strain
Y 33	Bakery strain
Y 80	Ale brewery strain
Y 86	Ale brewery strain
Y 261	Distillery strain
Y 304	NCYC 1211
Y 307	NCYC 1026
Y 311	NCYC 1083
Y 366	NCYC 366
ST*a*	Standard *a* mating strain
STα	Standard α mating strain
Saccharomyces carlsbergensis	
Y 41	Lager brewery strain
Y 43	Non-brewing hybrid strain
Y 90	Lager brewery strain, AJ 2094
Y 92	Lager brewery strain, AJ 2091
Y 93	Lager brewery strain, AJ 2036
Y 94	Lager brewery strain
Y 95	Lager brewery strain
Y 140	Lager brewery strain

TABLE 1 continued

NCYC stands for the National Collection of Yeast Cultures, located at the Brewing Industry Research Foundation, Redhill, Surrey; AJ, the Alfred Jørgenson Collection of Yeast Cultures, located in Copenhagen, Denmark. Yeast strains ST$\underline{a}$, ST$\underline{\alpha}$ and Y 43 were obtained from R. R. Fowell, Ewell Technical College, Epsom, Surrey.

Wallerstein Laboratories Nutrient-Agar (WLN; Green and Gray, 1950)

	g/litre
Yeast Extract (Difco)	4
Casitone (Difco)	5
Glucose	50
KH_2PO_4	0.55
KCl	0.425
$CaCl_2.2H_2O$	0.125
$MgSO_4.7H_2O$	0.125
$FeCl_3.6H_2O$	0.0025
$MnSO_4.4H_2O$	0.0025
Agar	20
Bromo-cresol green (Difco)	0.022

The salts were made up in solutions of one hundred times the final strength required. The other ingredients were made up to 500 ml, dissolved using a steam bath, and 10 ml of each salt solution was added. The medium was then made up to one litre, and portions (150 ml) dispensed in bottles (medical flats or 10oz bottles) and sterilised at 15 lb/in^2 for 15 min. Alternatively, the complete medium was obtained commercially (Difco) and 80 g of the powder were dissolved in one litre of distilled water by steaming. Portions (150 ml) were dispensed in bottles as before, then sterilised at 15 lb/in^2 for 15 min.

Brewers' Wort

The medium used for test-tube cultures, growth rate and fermentation rate determinations, estimations of head and flocculence and 1.5 litre tube-cultures was brewers' ale wort. This was produced by Allied Breweries at Burton, and was an aqueous extract of malted barley (ale malt) made at an extraction temperature of 65°C. The wort was then boiled with hops and filtered. It had a specific gravity of ca 1.060. This was diluted with water in the laboratory, to a sp. gr. of 1.040 ± 0.001. The diluted wort was filtered through kieselguhr (Filtercel; Johns Manville, Richmond) and then sterilised either by autoclaving at 15 lb/in^2 for one min, or by filtration through a sterile cellulose acetate membrane filter (47 mm diameter; pore size 0.22 μm; Millipore, London).

The ale wort used for the 20-litre fermentations was occasionally from the same source, but at other times it was obtained from the laboratory microbrewery. However the specification was the same for wort from both sources. Lager wort, used for 20-litre cultures, was produced in the microbrewery from lager malt; it had a final sp. gr. of 1.033 ± 0.001.

The wort used in the production-scale trials in the closed vessels (480 brl) was prepared as already described, and was diluted before inoculation with yeast to a sp. gr. of 1.038 ± 0.001. For production trials in the open vessels (60 brl and 168 brl) the wort had an original sp. gr. of 1.0365 ± 0.001.

Presporulation Agar

	g/litre
Glucose	20
KH_2PO_4	2
Yeast Extract (Difco)	5
Agar (Oxoid, No 3)	15

The medium was placed in a steamer to melt the agar. Portions (150 ml) of melted medium were dispensed in 10oz bottles (60 mm diameter x 150 mm) and further portions (15 ml) in MacCartney bottles (30 ml capacity). The bottles were then autoclaved at 15 lb/in^2 for 15 min. The MacCartney bottles containing molten agar medium were inclined while they cooled, to form slopes of solidified medium.

Sporulation Agar

	g/litre
Glucose	1
KCl	1.8
Sodium acetate (2 percent water)	8.2
Yeast Extract (Difco)	2.5
Agar (Oxoid No 3)	20

The medium was placed in a steamer to melt the agar. Portions (150 ml) were dispensed in 10oz bottles (60 mm diameter x 150 mm). Further portions (15 ml) were dispensed in MacCartney bottles (30 ml

capacity). All of the medium was autoclaved at 15 lb/in^2 for 15 min. The MacCartney bottles containing molten medium were inclined to produce slopes.

Sedimentation Broth (Chester, 1963)

	g/litre
Glucose	4
KCl	0.5
KH_2PO_4	1
$MgSO_4.7H_2O$	0.3
$(NH_4)_2SO_4$	2
Yeast Extract (Difco)	5

Portions (9 ml) of medium were dispensed in MacCartney bottles (25 ml) and autoclaved at 15 lb/in^2 for 5 min. A solution of calcium chloride containing 13.14 g/litre ($CaCl_2.2H_2O$) was prepared, Tyndallised, and a portion (one ml) was added aseptically to each bottle.

EXPERIMENTAL CULTURES

Test-Tube Cultures

Portions (10 ml) of the same batch of sterile brewery ale wort (sp. gr. 1.040 $\pm$ 0.001) in test-tubes (16 mm diameter x 150 mm) were each inoculated with a pinhead of yeast from an MYGP slope culture. The cultures were incubated statically for 72 h at 25°C, thoroughly shaken, and 1.0 ml of a uniform suspension was inoculated into each portion

(10 ml) of sterile wort (sp. gr. 1.040 $\pm$ 0.001) in tubes (16 mm diameter x 150 mm). The wort was again from the same batch. These cultures were incubated statically at 25°C for 24 h. They were shaken thoroughly and an absorbancy measurement was made on each culture using an EEL colorimeter, with a neutral density filter between the light source and the photocell. The cultures were then incubated statically for a further 72 h at 25°C and their final specific gravities were determined by the drop gravity method (Williams and Stringer, 1955).

After 96 h incubation, the cultures were cooled (4°C) overnight to sediment the yeasts, and the supernatants were discarded. The yeast sediments were then examined for degree of flocculence (p 43).

When the flocculence estimations had been completed, a portion (10 ml) of sterile wort (sp. gr. 1.040) was added to each tube containing sedimented yeasts, and the tubes were incubated overnight statically and examined for head formation.

Tube Cultures (1.5 litres)

Preparation of yeast inocula

Yeast strains to be tested were each inoculated into sterile ale wort (*ca* 400 ml; sp. gr. 1.040) in a conical flask (one litre capacity), and the cultures were incubated for 72 h to 96 h statically at 25°C. Strain Y 1 was included in each experiment as a control yeast. The cultures were centrifuged aseptically in weighed, sterile centrifuge bottles (one litre capacity) at 600 *g* in an MSE Major centrifuge, and the supernatants were discarded. The centrifuge bottles were reweighed,

the wet weight of yeast in each bottle calculated, and adjustments were made such that each bottle contained the same weight of yeast (between 4.0 and 5.0 g wet weight). The cells were then resuspended in one litre of sterile distilled water.

Fermentations in two-litre tubes (recommended by the European Brewing Convention; Cook, 1963)

Glass tubes (65 mm diameter x 900 mm length; two-litre capacity with a short side arm of 30 mm diameter, sealed with a Subaseal, size 53) were cleaned thoroughly and sterilised by allowing a 1% (v/v) Micro-clene solution (Soilax, Slough) to stand in the tubes overnight. Sterile cotton wool bungs were then placed in position to seal the open tops, and the sterilant solution was drained out through the tap at the base. Each yeast suspension was dispensed under nearly aseptic conditions. Portions (2 x 500 ml) were dispensed into two of the sterilised tubes, and boiled ale wort (one litre; sp. gr. 1.060 $\pm$ 0.004) was added to give an approximate overall sp. gr. of 1.040. The contents of the tubes were mixed by shaking, and a sample (two ml) was then withdrawn from each tube through the Subaseal using a sterile syringe.

These samples, and all others taken twice daily throughout the fermentation period, were tested for specific gravity by the equilibrium drop technique and for cell count. In addition, the yeast head produced on the fermenting liquid in each tube was measured (cm) and selected samples were plated out in WLN containing actidione (p 45) to test for bacterial infection.

When fermentation was judged to be complete, that is, when the rate of drop in specific gravity was less than 0.0005 units in 24 h, or when

the specific gravity had fallen below 1.012, the yeast crop from each tube was harvested. Total wet weight yields for each test strain and the control strain Y 1 were determined.

Canister Cultures (20 litres)

Preparation of yeast inocula

Each yeast under test was inoculated into portions of sterile ale or lager wort (3 x 600 ml portions of ale wort, sp. gr. ca 1.040, or 4 x 600 ml portions of lager wort, sp. gr. ca 1.033) in conical flasks (one litre) and incubated statically at 25°C for 72-96 h. Yeasts Y 1 and Y 140 were control strains used for ale and lager fermentations respectively.

The cultures were centrifuged aseptically in weighed, sterile centrifuge bottles (one litre) at 600 g on an MSE Major centrifuge, and the supernatant was discarded. The centrifuge bottles were reweighed, the wet weight of yeast in each one was calculated, and each test and control yeast slurry was distributed into two sterile conical flasks (250 ml), to give the same wet weight in each flask (between 25 and 30 g for ale fermentations, and 40 to 50 g for lager wort fermentations). Sterile potassium dihydrogen orthophosphate solution (2%, w/v; ca 50 ml) was added to each flask and the yeast suspensions were stored for up to 24 h at 4°C until used.

Fermentations in canisters

The stainless steel canisters used (Cornelius, Bedford) had a 30-litre capacity and were cylindrical (300 mm diameter x 650 mm height) and

had a plastic lid fitted with a pressure release valve. They were sterilised by steaming for two hours or autoclaving at 15 lb/in^2 for 15 min, with the release valve open. Sterile wort (ca 20 litres) of the appropriate type (ale wort, sp. gr. 1.040, or lager wort, sp. gr. 1.033) was introduced aseptically into four canisters and two canisters were inoculated with the test and two with the control strain of yeast. They were then placed in a water bath maintained at 21°C for ale fermentations or 14°C for lager fermentations. Sterile samples were taken immediately after inoculation, and then twice daily, and these were examined for specific gravity (equilibrium drop technique), yeast count (haemocytometer), infection level (by plating in WLN agar containing actidione) and yeast stability (by plating on WLN agar).

When the fermenting cultures had reached a sp. gr. of 1.012 $\pm$ 0.001 for ale fermentations or 1.008 $\pm$ 0.001 for lager fermentations, the canisters were removed from the water bath and placed in a cold room (4°C) to chill the contents and sediment the yeast. After 48-72h cooling at 14°C, finings (Saville, Aldridge) were added to a concentration of 7 ml/l. The beers were left for 24 h at 14°C, then removed from the yeast sediment into a second stainless steel canister (18 l capacity; Cornelius, Bedford) and stored at 14°C. Carbon dioxide gas (Air Products, Darlaston) was added to each canister (ca 2.8 g/l), the beers were chilled again (14°C) for 24 h and tasted against the appropriate control beers, using the three glass preference technique (p 46).

PRODUCTION SCALE FERMENTATIONS

Three yeasts have been tested in production-scale fermentations, namely H 10, H 1431 and H 1465. The details of the trials are given in Table 2. Four different scales of fermentation have been carried out (27, 60, 168 and 480 barrels).

Preparation of Yeast for Inoculation

A step-up procedure was used to grow the hybrid yeasts for the trials. The final culture (5 l) of the strain, incubated in wort of the appropriate starting gravity (see Table 2) at 25°C for 48-72 h, was introduced aseptically into a yeast propagation vessel (capacity eight barrels for open vessels, 35 barrels for closed vessels). Then sterile wort of the specified gravity was added, (six barrels for open fermentations and 30 barrels for closed fermentations) and the cultures were allowed to grow for 48-72 h at 21°C. Control yeasts used in the fermentation trials were obtained from the brewery fermentations, and they were pressed to remove water (Johnson Press), stored at 5.5°C until needed, and slurried with an equal volume of water prior to inoculation into fresh wort.

Fermentations in Open Vessels

The propagated cultures of hybrid yeasts and slurries of control strains were transferred into ale wort of sp. gr. of ca 1.0365, at 18°C to give an initial yeast count of between nine and ten million cells per ml. The fermentations were allowed to proceed, and 'heads' of yeast

TABLE 2

Specifications for, and volumes of, trial and control production-scale fermentations

No of Trials	Specified initial specific gravity of wort	Specified initial yeast cell count x 10^6/ml	Trial fermentation		Control fermentation		Type vessels used
			Hybrid strain	Volume of ferm^{n}s in brls	Control strain/s	Volume of ferm^{n}s in brls	
1	1.0365	9-10	H 10	27	Y 7 + Y 9	168	Open
2	1.0365	9-10	H 1431	60	Y 7 + Y 9	60	Open
2	1.0365	9-10	H 1465	60	Y 7 + Y 9	60	Open
6	1.038	6-9	H 1431	480	Y 1	480	Closed

1 barrel (brl) is equivalent to 163.65 litres

were removed by skimming when necessary, between 44 and 66 h after the start of fermentation. However, heads produced on trial fermentations of strain H 1465 were inadvertently not removed.

The beers were run-off from the fermentation vessels, clarified using finings (ca 1.9 l per brl; Saville, Aldridge) and the bitterness was then adjusted to 31 ± 2 EB Units. They were then transferred into stainless steel barrel-shaped vessels of ca 41 l capacity, which were stored at 13°C before analysis and taste trials. When fermentations were repeated yeast was harvested from the first fermentation, and pressed and treated in the same way as the control strains.

Fermentations in Closed Vessels

The propagated yeast cultures (control and hybrid strains) were inoculated into the vessels containing ale wort (480 brl; sp. gr. ca 1.038) at 16°C, to give a starting cell count of between six and ten million cells per ml. The fermentations were allowed to proceed, the maximum temperature being controlled at 23°C. Mixing was effected using a screw-rousing system. When fermentation was judged to be complete (at a sp. gr. of 1.0125 ± 0.001) the beer was run off, and the yeast transferred to another vessel until required for the next fermentation. A series of six fermentations was carried out.

The beers were centrifuged (120 or 250 brl/h; Alfa Laval, Brentford) and fined (1.2 l/brl; Saville, Aldridge), run off from the yeast and finings, and the bitterness adjusted to 24 ± 3 EB Units. They were then transferred into 41 litre vessels, and stored pending analysis and taste trials.

Measurements Conducted on Samples from the Fermentations

All production-scale fermentations were sampled at regular intervals (every 4-8 h) and the samples were examined for yeast count, specific gravity and bacterial infection. Additional observations were made on the open fermentations; head formation was observed throughout the period, and the total yeast crop (wet weight) and reproduction ratios ($\frac{\text{weight of crop}}{\text{weight of yeast inoculated}}$) were estimated.

Analyses on Beers

Samples were taken from the 41-litre vessels and analysed for alcohol concentration, bitterness, colour, diacetyl, dimethyl sulphide, nitrogen content (α-amino and total), pH value, total polyphenol content, original specific gravity and present gravity, and a range of volatile components.

OBSERVATIONS ON EXPERIMENTAL CULTURES

Average Cell Volume

Yeast strains to be tested were incubated statically for 72-96 h in ale wort (10 ml; sp. gr. 1.040) at 25°C. Since these cultures were in the stationary phase of growth, bud formation should be minimal. They were mixed thoroughly and samples were diluted if necessary, before microscopic examination. The mean long diameter and mean short diameter of a hundred randomly selected cells were measured using either a micrometer eyepiece graticule (Vickers, York) or a screw micrometer eyepiece

(Zeiss), which had been calibrated using a stage micrometer (Watson, Barnet). These two techniques gave similar calculated volume ($\pm$ 5%) when tested using the same yeast culture. The mean long radius and mean short radius were calculated for each yeast strain under test. A Vickers microscope was used. The average cell volume for each yeast strain was calculated from $\frac{4}{3}\pi ab^2$, where a is the mean long radius and b, the mean short radius.

Comparisons of cell sizes could only be made between yeasts grown in wort derived from the same batch. Different batches introduced variations in calculated volumes. The standard deviation of measurements made on replicate samples of one strain in wort from a single batch, expressed as a percentage of the mean, is $\pm$ 10%.

Growth Determinations

The absorbance of test tube cultures, after incubating for 24 h (p 34) and mixing thoroughly, was measured using an EEL colorimeter, with a neutral density filter between the light source and the photocell. A test tube of ale wort (10 ml; sp. gr. 1.040) was used as a blank. When measurements were being made on cells suspended in any other medium, that medium was used as the blank.

Specific Gravity

This method was originally described by MacDougald(1909), later modified by Ault (1954), and improved by Williams and Stringer (1955), It involves setting up a series of test tubes containing sucrose solutions

of known specific gravities ranging from 1.005 to 1.040, and containing mercuric chloride (0-5 g/l; $\frac{1}{20}$ of concentration of sugar solution) to prevent infection. A drop of a solution of unknown gravity (a wort, a beer, or a fermenting wort), is then added to selected tubes. After falling a distance of about 20 mm, the drop will either continue to fall, or rise. If it falls, it has a higher specific gravity than the surrounding liquid, if it rises, a lower one.

Flocculence

A method adapted from the technique of Gilliland (1957) was used to evaluate the flocculating ability of all of the hybrid yeasts. Two parent strains namely Y 1, which is a moderately flocculent ale yeast, and Y 80, which is a very flocculent ale yeast, were included with the strains under test. The yeast sediments from 96 h test-tube cultures (p 34) were examined after shaking. Yeast strains which produced similarly granular sediments to Y 80 were considered to be in group IV, the very flocculent group, and those which resembled Y 1 were included in the flocculence group III. Others which produced less granular sediments than strain Y 1 but which still showed some degree of floc formation were classified under group II. Strains which gave sediments of totally non-granular appearance, that is, were not flocculent, were included in group I. The flocculence group was shown to be a stable characteristic for any one strain.

Head Formation

The method used is an adaptation of the Harris and Watson technique (Harris and Watson, 1971). Yeast sediments from the flocculence test were resuspended in fresh ale wort (10 ml; sp. gr. 1.040) in test tubes (16 mm diameter x 150 mm length) as described before (p 34) and incubated overnight at 25°C. Strains Y 1 and Y 80 were again used as reference yeasts. Strain Y 1 is a head-forming strain and is said to form a ++ head; strain Y 80 forms no head and is said to be a - head former. The heads of yeast formed on the cultures of the test strains were compared with those of the control yeasts, and head-forming ability was assessed on the scale -, +, ++, +++ by measuring the head height. The nature of each yeast head, whether it had a foamy or yeasty texture, was taken into consideration in the assessments.

Yeast Cell Count

Cell counts were carried out using a Fuchs-Rosenthal haemocytometer slide. Cell suspensions were diluted to between one and three million cells per ml. A coverslip was slid into position on the counting chamber, which had a depth of 0.1 mm, so that a tight seal was obtained on either side. Each section or chamber of the haemocytometer slide was marked out with a grid of lines, defining nine squares (three by three) with side length 1 mm, each divided into 16 small squares. A sample of the diluted yeast suspension was introduced into the counting chamber using a capillary tube. Yeast cells, which were lying within the limits of any three of the nine large squares, were counted, and the number of cells per ml of original suspension was calculated.

Wet-Weight Yield

At completion of 1.5-litre fermentations in the tubes (two litre), an estimate of total crop for each yeast strain under test was made as follows. The contents of each tube were mixed to resuspend any yeast adhering to the side of the glass tube, then the contents were drained out and centrifuged at 700 *g* for 15 min in a weighed centrifuge bottle (one litre) on an MSE Major centrifuge. The supernatant was decanted and the remainder of the contents from that tube was then added, and centrifuged as before. The supernatant was discarded again, the bottle and centrifuged yeast was weighed, and the total crop from each tube was determined.

Bacterial Cell Count

Samples (one ml) from the 1.5-litre and 20-litre fermentations were suspended in molten WL Nutrient agar containing actidione (15-20 mg/litre). After pouring, the plates were cooled until the agar had set. They were incubated at 25°C for 48-72 h and examined for bacterial and wild yeast colonies.

Yeast Stability

Samples from the 20-litre fermentations or from test-tube cultures of hybrids were diluted and plated (0.1 ml) on WL Nutrient agar to give 50 to 100 colonies per plate. Plates were examined after 72 h incubation at 25°C, and estimates were made of the stability of the yeast strain from the proportion of abnormal yeast colonies produced. If less than 15 per

cent abnormal colonies were apparent, the yeast was considered to be acceptably stable. Parent strains used for hybridisation produce up to 15 per cent abnormal colonies (see Table 13).

Tasting Trials

The flavour of a beer produced using a hybrid yeast strain was compared with that of a beer fermented by the appropriate control strain, namely Y 1 for ales, Y 140 for lagers. A number of tasters (up to 12) were requested either to compare two glasses of beer, one a trial, the other a control, and rank them according to preference, with relevant comments, or to carry out a three-glass triangular test (Bengtsson, 1953) on the two beers. The results were analysed statistically.

HYBRIDISATION PROCEDURES

Sporulation Technique

Yeasts were streaked on to presporulation agar plates, incubated at 20°C for 48 h, then restreaked thickly from the plates on to sporulation agar slopes, and incubated at 20°C. The slope cultures were examined periodically for mature asci, after incubation for 48 h and up to 168 h after inoculation.

Some of the parent strains sporulated poorly and/or produced only one- and two-spored asci, and it was difficult to assess levels of sporulation in these strains using microscopic examination of a prepared wet-mount slide. With these yeasts, a spore stain was employed. This

was a modification of the method devised by Scaeffer and Fulton (1933). A suspension of the yeast in water was heat-fixed to a microscope slide and then flooded with Malachite green solution (5% w/v; Hopkin and Williams). The liquid on the slide was heated to steaming point over a flame for 30 sec, but was not allowed to dry out. The slide was then washed under running water for 30 sec, and flooded with safranin solution (0.5% w/v; Gurr) for 10 sec. The slide was finally water-washed, dried and the preparation was examined under a microscope, using an oil-immersion lens. Spores stained green and vegetative cells deep pink.

Isolation of Potential Mating Strains from Asci

Three different techniques were used to split open asci and separate spores from one another. The first two are 'mass isolation methods' (Lindegren and Lindegren, 1943a).

1. Broth and plating technique

Liquid medium (MYGP; 10 ml) was added to the organisms (asci and vegetative cells) on sporulation agar slopes. The cells were washed off into the liquid, and the broth cultures incubated for 18 h at 25°C. Suitable dilutions were prepared in sterile quarter-strength Ringer's solution (containing per litre; 2.25 g NaCl, 0.105 g KCl, 0.12 g $CaCl_2.2H_2O$ and 0.05 g $NaHCO_3$; pH 7.0; Davis, 1956) to which one per cent Tween 80 (Hopkin and Williams, Chadwell Heath) had been added. The dilutions were mixed thoroughly, and portions (0.1 ml) were plated out on WLN agar plates, and incubated at 25°C for 72 h.

Small colonies and those of unusual colonial morphology or colour were picked off, and streaked on to sporulation agar plates. Samples of

the parent strains were also streaked from 72 h plate cultures on WLN agar, on to sporulation agar plates from the same batch of medium. All of the plates were incubated at 20°C. The culture of each parent strain was examined for sporulation after 48 h and every further 24 h up to 168 h. When the parent strain was sporulating, the cultures derived from the small and unusual colonies of the same yeast were examined. If these cultures were not capable of sporulation after 168 h incubation, they were considered to be potential mating strains, and were inoculated on to MYGP agar slopes, incubated at 25°C for 72 h and stored at 4°C.

2. Enzymic and plating technique

The mixture of asci and vegetative cells on sporulation agar was suspended in quarter-strength Ringer's solution (1 ml) to give a concentration of 10^7 to 10^8 organisms/ml. A commercial solution of helicase (0.01 ml; Microbio, London) which is a mixture of enzymes (Holden and Tracey, 1950), was added to each suspension and the suspensions were incubated at 20°C for 18 h. The suspensions were then diluted in quarter-strength Ringer's solution containing Tween 80 (1% w/v) and were plated as described above (p 47). Small colonies were selected and examined for sporulating ability.

Potential mating strains were again inoculated on to MYGP agar slopes, grown up at 25°C for 72 h, and stored at 4°C.

3. Micromanipulation technique

This method was used only for strains of yeasts which sporulated poorly and/or produced only sterile mating strains by the other techniques.

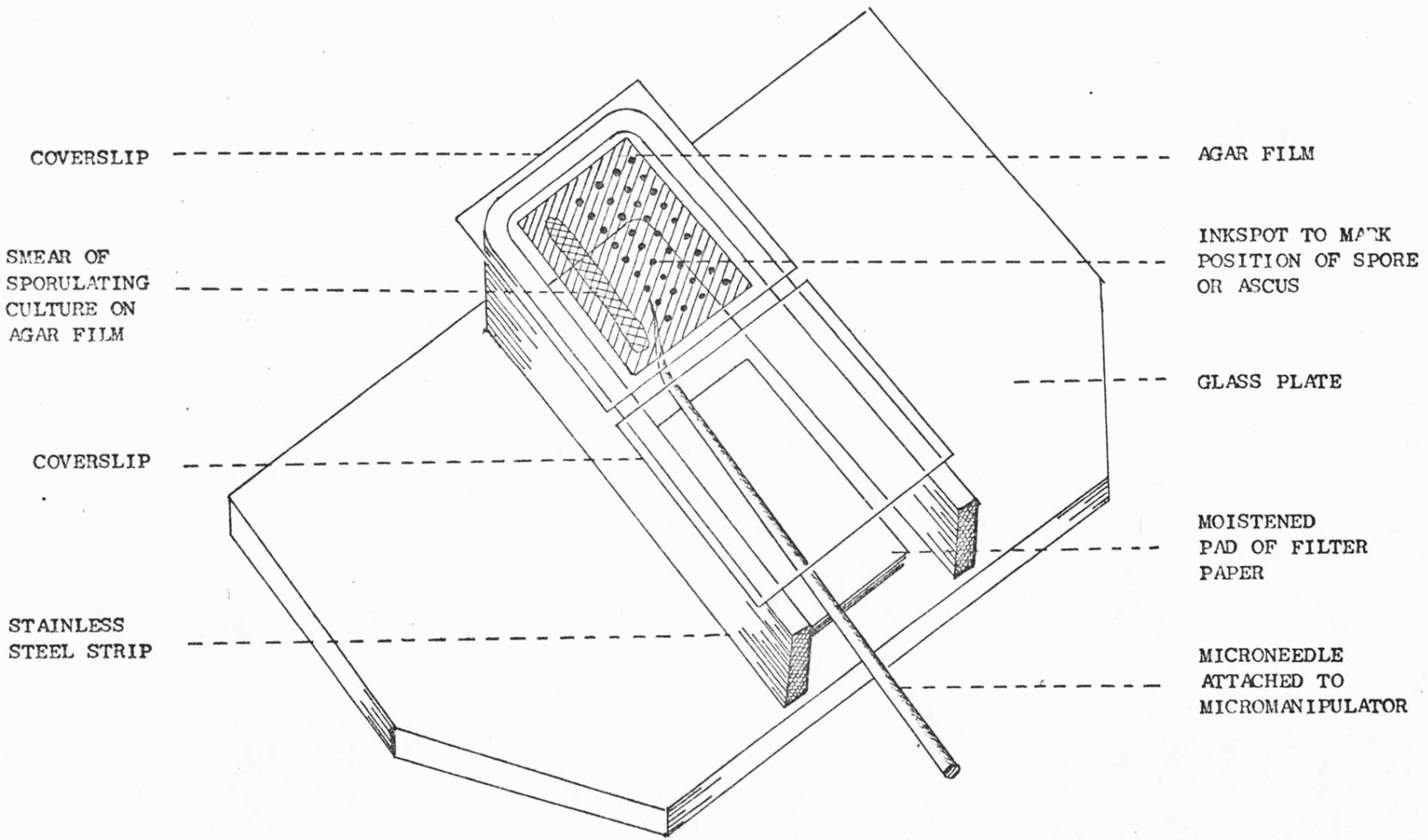
Preparation of yeasts for micromanipulation: Strains Y 9, Y 11, Y 41, Y 93 and Y 140 were induced to sporulate after 168 h incubation on sporulation agar. From each sporulating culture, a suspension of cells including asci was prepared in quarter-strength Ringer's solution (10^7 to 10^8 cells/ml). Helicase solution (0.01 ml) was added to the suspensions, which were then incubated for 18 h at 20°C.

Preparation of agar film: The technique used was that used by Fowell (1969). A rectangular glass coverslip (22 mm x 33 mm; 0.2 mm thick) was dipped in methanol, flamed and placed on a hot plate at 50°C. Two strips of glass (ca 5 mm x 22 mm) were cut from a further coverslip, and were flamed and placed along the short edges of the one on the hot plate. A second coverslip (22 mm x 32 mm) was sterilised by flaming and placed on top of the strips, so that this overlapped the lower one by about three mm along one long edge. Molten MYGP agar, at ca 50°C, was introduced at the other long edge using a sterile Pasteur pipette. The two coverslips were then lifted off the hot plate with sterile forceps and placed in a sterile petri dish to cool. When cool, the top coverslip and side strips of glass were carefully removed using forceps and a scalpel, sterilised by dipping in methanol and then flaming. The sterile agar film (ca 0.2 mm thick) was kept in the petri dish until required.

Preparation of the moist chamber: The moist chamber was prepared as described by Fowell (1969); an adaptation of his diagram is shown in Figure 1. Nail varnish (Boots Pure Drug Co., Nottingham) was used to secure a U-shaped stainless steel strip, (ca 15 mm deep) to a glass plate.

FIGURE 1

MOIST CHAMBER USED FOR MICROMANIPULATION OF ASCI



The prepared coverslip, agar film downwards, and a second sterile coverslip were also secured to the top surface of the metal strip using varnish, as shown (Figure 1). A pad of filter paper, moistened with sterile water, was placed in the chamber to keep the air moist around the agar film during micromanipulation. The enzyme-treated mixture of asci and vegetative cells was streaked on to the edge of the agar film, and ink dots, in rows of four, were marked on the upper surface of the coverslip, as shown in Figure 1.

Micromanipulation: Rupture of selected asci was carried out in the moist chamber using a microneedle, (end diameter, 30 to 100 μm), attached to a DeFonbrune pneumatic micromanipulator, combined with a microscope which had a fixed slide stage and a moving objective head. The needle was carefully introduced into the moist chamber which was on the microscope slide stage. Its level and position were adjusted, watching the progress under the microscope, until the tip was just touching the surface of the agar film, near the smear of asci and vegetative cells. Each ascus was selected from the mixture, touched with the tip of the needle, and pulled away from the smear along the surface of the agar film to the first row of ink dots marked on the coverslip. Close to the nearest dot in that row, the ascus was gently rolled about on the agar until it had ruptured. The spores were separated and each one was placed by one ink dot in the row of four. When the component spores of a two-spored ascus could not be separated, the complete ascus was transferred to a marked position. The low viability of spores in poorly sporulating strains could mean that only one of the spores would germinate. A note was kept of the origin of each isolate at each ink dot.

Isolation of cultures derived from spores and asci: After micromanipulation, the coverslip, with the agar film on it, was carefully removed from the moist chamber and secured, agar film upwards, to the inside of the lid of a sterile petri dish, using varnish. Filter paper, moistened with sterile water, was placed in the base of the dish, to prevent desiccation of the agar film, and the whole dish was incubated overnight at 20°C. The agar film was then cut, with a sterilised scalpel, into small squares, each with a single germinated spore or ascus on it. Each square of agar was inoculated into a test-tube (16 mm x 150 mm) of sterile ale wort (10 ml; sp. gr. 1.040). Spores which had apparently not germinated were also inoculated into tubes of sterile wort. The wort cultures were incubated statically at 25-28°C for 48-72 h until growth was evident. Portions (0.1 ml) of suitable dilutions of the cultures were then plated out to give 50 to 100 organisms per plate. The plates were incubated at 25-28°C for 72 h and typical colonies were selected from each plate as potential mating strains. These were streaked on to MYGP agar slopes, incubated at 25°C for 72 h, and stored at 4°C. All cultures were also checked for sporulating ability.

Determination of Mating Ability

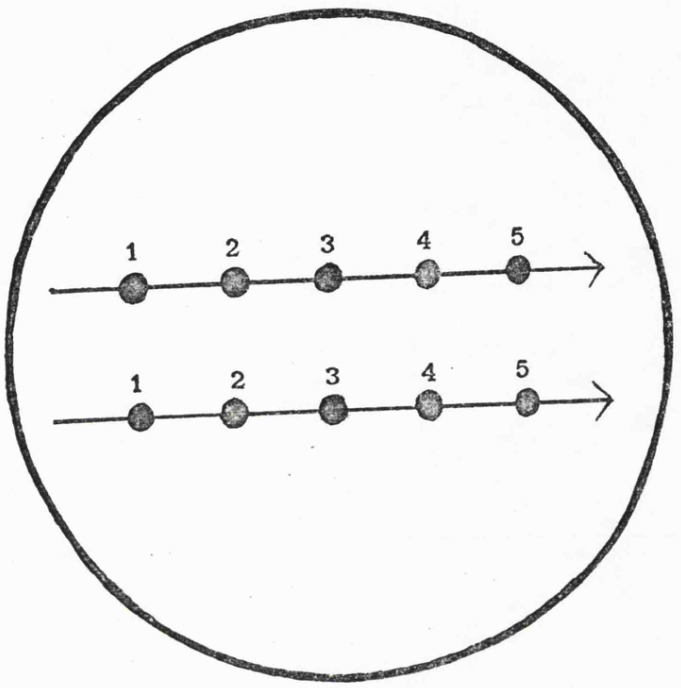
All of the cultures which had been isolated as potential mating strains were examined for mating type. Reference strains ST$\underline{a}$ and ST$\underline{\alpha}$ were streaked on to a WLN agar plate (see Figure 2, top) and each strain under test was point inoculated on to the two streaks. The plates were then incubated at 25°C for 48-72 h. Mating of the strain of unknown mating type with ST$\underline{a}$ and/or ST$\underline{\alpha}$ was confirmed after this time, either

FIGURE 2

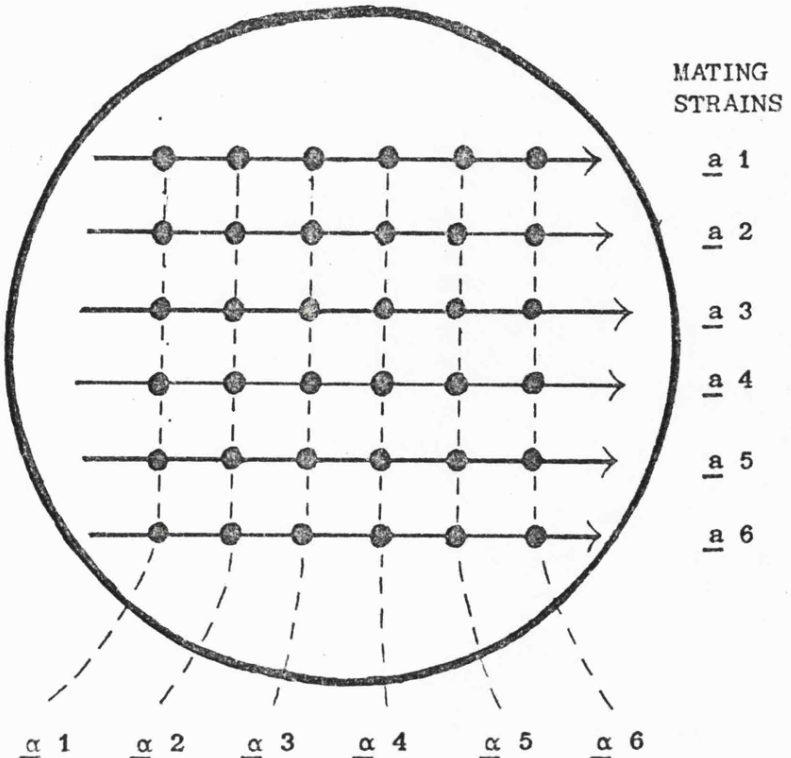
SCHEMATIC REPRESENTATIONS OF PLATES USED FOR MATINGS

1. ARRANGEMENT OF COLONIES ON PLATES TO DETERMINE MATING TYPES OF POTENTIAL MATING STRAINS

1, 2, 3, etc are potential mating strains of unknown mating type



2. ARRANGEMENT OF COLONIES ON PLATES TO HYBRIDISE MATING STRAINS OF KNOWN MATING TYPE



⟶ indicates that the cultures are streaked on the agar

● indicates that the culture is point inoculated on to the streak

microscopically by looking for zygote formation, or by testing for sporulating ability after mating. A sporulating product of a cross between a non-sporulating isolate and a standard strain would indicate that the two had mated successfully. Therefore all mating strains were designated a, α or a/α in mating type. Strains which would not mate with either STa or STα were considered to be sterile.

Hybridisation

Six different mating strains of the same mating type or of the combined mating type were streaked on to a WLN agar plate. Each of six mating strains of the opposite or combined mating type were point-inoculated on to each of the six original streaks, to give a total of 36 possible mating zones on each plate (Figure 2, bottom). The plates were incubated for 72 h at 25°C and then examined for positive mating, again either by microscopic examination or by testing the yeasts from the mating zones for sporulating ability.

Not all of the mating strains isolated and used to prepare hybrid yeasts were non-sporulating, nor were they all derived from spore isolates. It was found that some of the poorly sporulating parent strains of yeast were themselves able to mate with one or other of the standard strains STa and STα, and with selected mating strains derived from the parent strains. Mating was assessed by zygote formation, and a marked improvement in the ability to sporulate after hybridisation. In addition, some of the mating strains isolated by micromanipulation were capable of sporulating, and when crosses were attempted with these yeasts, mating

could only be confirmed by zygote formation or a large improvement in sporulating ability of the 'hybrid', compared with that of its parent strain.

Isolation of Hybrid Strains

When mating between two strains had been confirmed, a sample was taken aseptically from the appropriate zone on the plate. In the early stages of the hybridisation programme, it was inoculated into a test-tube (16 mm x 150 mm) of sterile ale wort (10 ml; sp. gr. 1.040) incubated at 25°C, subcultured on three consecutive days into fresh portions of wort (10 ml; sp. gr. 1.040) and incubated at 25°C in between subcultures. This was an adaptation of the method of Fowell (1969). Each hybrid was then streaked on to a WLN agar plate, incubated for 72 h at 25°C and examined. If the culture was relatively pure, a typical colony was picked off and inoculated on to an MYGP agar slope; if this plate culture appeared to consist of a number of strains, a series of subcultures on WLN agar plates, incubating at 25°C for 48-72 h between subcultures was used to isolate and select the fastest growing hybrid. Later in the hybridisation programme, subcultures on WLN agar plates, incubating as before, were adopted as the method to isolate the hybrid strain. This technique also gave an indication of the genetic stability of the new hybrid.

OTHER OBSERVATIONS MADE ON SELECTED YEAST STRAINS

Determination of Specific Growth Rate

Preparation of inocula

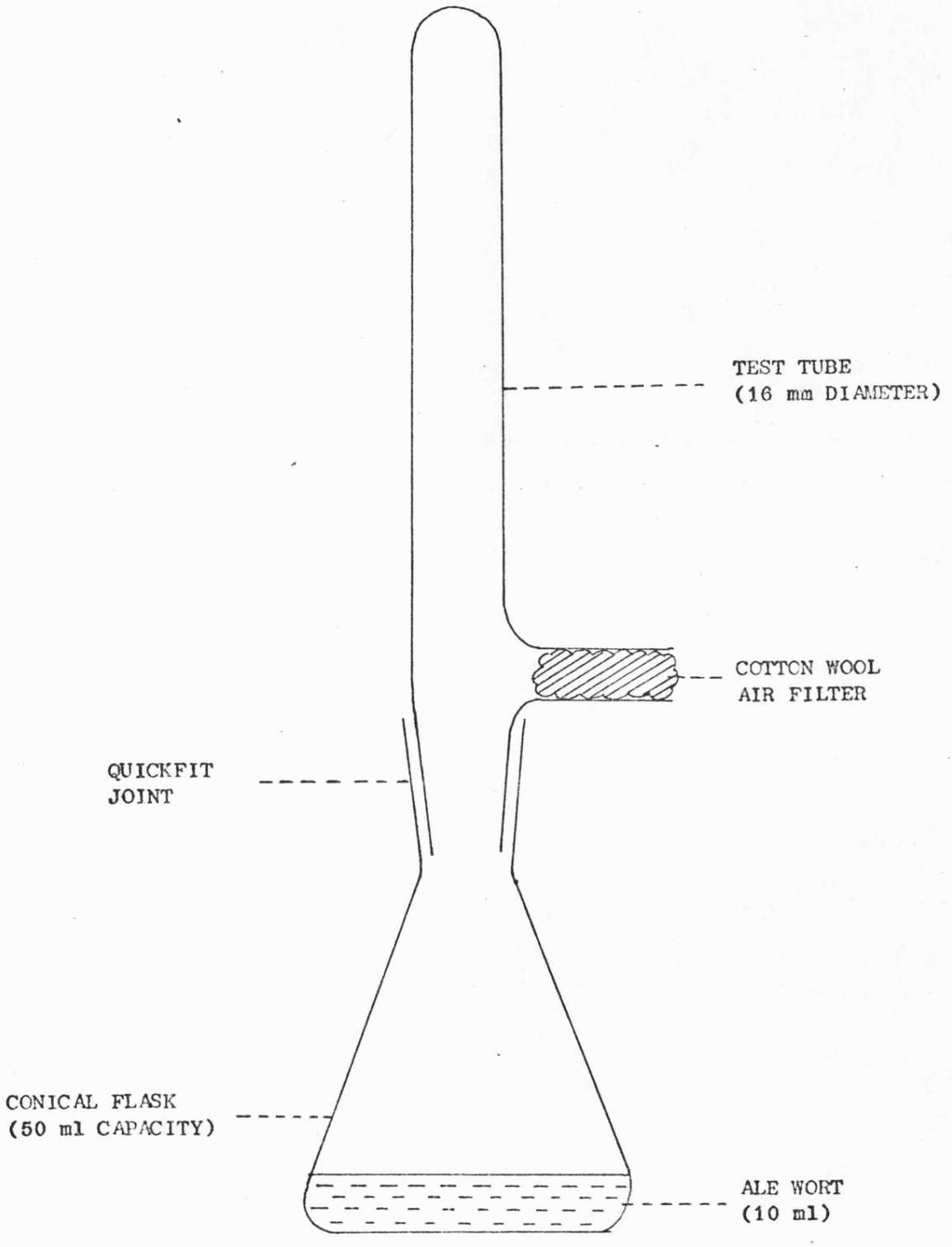
Yeast strains to be examined were inoculated into sterile ale wort (10 ml; sp. gr. 1.040) in MacCartney bottles (30 ml) and the cultures incubated statically at 25°C for 24 h, and then mixed thoroughly. Strain Y 1 was always used as a control yeast. A portion (0.1 ml) from each culture was inoculated into another bottle of wort (10 ml; sp. gr. 1.040) from the same batch, and incubated statically at 25°C overnight. The cultures were then centrifuged on a BTL bench centrifuge (3200 g) and the supernatants discarded. The yeast sediments were resuspended in sterile phosphate buffer (Sorenson's, 0.067 M-potassium dihydrogen orthophosphate solution and 0.067 M-disodium hydrogen orthophosphate solution; pH 5.0) and the absorbancy readings of each suspension were adjusted to 0.1 measured on an EEL colorimeter using a neutral density filter. This corresponds to a cell count of one to three million cells per ml.

Determination of growth rates

A portion (0.1 ml) of each yeast suspension was transferred to the apparatus shown in Figure 3, containing ale wort (10 ml; sp. gr. 1.040) that had been sterilised by filtration through a cellulose acetate membrane filter (47 mm diameter; pore size, 0.22 μm; Millipore). Triplicate flasks were set up for each yeast strain under test, and were incubated at 25°C on an orbital incubator, shaking at 150 rev/min, with stroke length

FIGURE 3

APPARATUS FOR MEASUREMENT OF SPECIFIC GROWTH RATE AND ESTIMATION OF DURATION OF LAG PHASE



3.5 cm. After 16 h incubation, absorbancy readings were taken every 30 min, by inverting the flask and inserting the tube section into the tube holder of an EEL colorimeter. A control flask, containing uninoculated ale wort from the same batch was used as a blank. Every set of three absorbancy readings at one particular time was averaged, and the averages were plotted against time on semi-logarithmic graph paper, with the absorbancies on the logarithmic axis.

The specific growth rate (μ) for each strain of yeast was determined from the slope of the plot using the formula:

$$\mu = \frac{\log_e (x_1 - x)}{t_1 - t}$$

where x is the absorbance at time t, and x_1 that at time t_1. An example of the plot can be seen in Figure 15 (p 106). An estimate of the duration of the lag phase in a culture, compared with that of the control strain Y 1, could be made from a linear plot of the results; for an example see Figure 16 (p 108). An arbitrary level was taken, at an EEL reading of 0.1, below which the yeasts were considered to be in the lag phase of growth. Then the time after inoculation, at which each plotted line crosses that level, gives an estimate of length of lag phase compared with that of the control yeast Y 1.

Determination of Fermentation Rate

In each experiment, up to six strains of yeast were tested for rate of fermentation of a substrate, using a Gilson Differential Respirometer.

Preparation of yeast suspensions

The selected strains were each inoculated into a test-tube (16 mm x 150 mm), containing ale wort (15 ml; sp. gr. 1.040) that had been filtered through a sterile cellulose acetate membrane filter (47 mm diameter; pore size 0.22 μm; Millipore), and the tubes were incubated statically at 25°C overnight. These cultures were mixed thoroughly, portions (2 x 0.05 ml, 2 x 0.1 ml and 2 x 0.25 ml) of each were inoculated into test-tubes of sterile filtered wort (6 x 15 ml; sp. gr. 1.040), from the same batch, and the replicate tubes of each yeast strain were incubated as before. The absorbancies of the cultures were measured on an EEL colorimeter, and two cultures of each yeast were selected which had an absorbancy reading of 1.0 units ± 0.15 units. Over this range of absorbancy, the cultures were estimated to be in the logarithmic phase of growth (see Figure 15, p106). The selected cultures were transferred aseptically to sterile centrifuge tubes (30 ml capacity), the two cultures of each strain being mixed. The combined culture was centrifuged at 3200 g for 15 min on a BTL bench centrifuge. The supernatants were discarded, and the sedimented yeasts were resuspended in Sorenson's phosphate buffer (pH 5.0; 0.067 M-potassium dihydrogen orthophosphate and 0.067 M-disodium hydrogen orthophosphate solutions), to give an EEL reading of 1.0 which is equivalent to a count of between 10 and 25 million cells per ml, depending on the yeast strain used.

Dry weight determinations

Portions (2 x 10 ml) of each yeast suspension were filtered through

weighed cellulose acetate membrane filters (47 mm diameter; pore size 0.45 μm; Millipore) that had been dried to constant weight (40°C overnight). After washing the yeasts on the membrane filters with glass-distilled water, and filtering the washings, the membranes were dried overnight at 40°C, cooled to room temperature in a desiccator, and reweighed. The mean dry weight (per ml), of each yeast suspension was calculated from the duplicate estimations.

Preparation of respirometer flasks

The respirometer flasks (18 ml capacity), each with a single side-arm and a gas-venting stopper, were carefully cleaned and dried. Substrate solutions added to the flasks were always at 1.5 times the concentration required in the experiment, because addition of the yeast suspension restores that concentration. These substrates were ale wort (sp. gr. 1.060), glucose (6% w/v), maltose (6% w/v) or maltotriose (1.5% w/v; see p 68).

Two respirometer flasks were set up for each yeast strain under test, with yeast suspension (1 ml) added to the side-arm of both flasks, and phosphate buffer (2 ml; Sorenson's; pH 5.0) added to the main compartment of one (the yeast control flask), and the appropriate substrate solution (2 ml) to the main compartment of the other (the yeast test flask). A substrate control flask was also prepared, with phosphate buffer solution (1 ml) in the side-arm and the substrate solution (2 ml) in the main flask.

The thermobarometer flask (250 ml capacity) of the respirometer was removed, and a volume of distilled water was added equal to the total volume of liquid in all of the test flasks. Thus, if six yeasts were

being tested, the volume to add will be ((6 x 2) + 1) x 3, that is, 39 ml. It was then replaced on the respirometer, with its side-arm stopper in the venting position.

The necks and side-arm stoppers of all of the respirometer flasks were lightly greased (Vaseline) and the apparatus was assembled with the side-arm vents open. The complete apparatus was then gassed out with oxygen-free nitrogen (Air Products), for ca 30 min, with all of the flasks submerged and shaking in the water bath (ca 60 strokes per min; stroke length, 3.0 cm) at 21°C or 25°C, and with the valves to the manometers closed. The gas flow was stopped, the manifold valve and side-arm stoppers were turned to the closed position, and the flasks were shaken for a further 10-15 min. Then the contents of the side-arm of each flask were carefully tipped into the main compartment of the flask, and mixed by tipping backward and forward between the arm and the flask. The flasks were then put back in the water bath, shaking was continued, and the manometer head micrometers were set to a reading of 450. The manometer valves were then opened.

Determination of fermentation rates

The flasks were left to equilibrate for 10 min, and the manometer valves were closed and then opened again. This was done to bring the level of the liquid in the manometers back to the original level shown by a marker, set at the equilibrium level, when the fluid in both arms of each manometer was at the same height. At intervals (5 min) the level of fluid in each manometer was brought back to the marker position by screwing the manometer head micrometer in the appropriate direction,

and a reading of the volume was taken from the micrometer. The reading dropped as gas was evolved from each respirometer flask. The figures obtained for the yeast test flasks were used to derive the gas volumes produced. Corrections were made for changes in the substrate controls and yeast control flasks. Graphs were plotted of volume of gas evolved against time, and the rate of evolution of carbon dioxide (ml/min/mg dry weight) for each yeast under test was calculated. The coefficient of variation of the precision of the method, determined from six sets of replicate experiments, was $\pm$ 2.0%.

Sedimentation Rates

A simplified method, based upon that of Chester (1963), was adopted substituting visual examination of the sedimentation behaviour for an absorptiometric method.

Preparation of cultures

The yeast strains to be tested, including strains Y 1 and Y 80, were inoculated into portions (10 ml) of Chester broth (p 33) and incubated for 96 h at 25°C, the cultures being vigorously shaken every 24 h. After 96 h, the cultures were again shaken, and were then set up against a dark background.

Estimation of sedimentation rate and designation of flocculence group

All cultures were compared with those of control strains Y 1 and Y 80. If they showed a similar rate of sedimentation to Y 80, which is a strongly

flocculent yeast, they were considered to belong to group IV; if, in behaviour, they were similar to Y 1, they were considered to be in group III. Yeasts which sedimented more slowly than Y 1, but still formed flocs, were classified in group II, and the non-flocculent strains were considered to be in group I.

ANALYTICAL METHODS

Protein Determinations

All glassware used for these determinations was cleaned with chromic acid and rinsed twice with glass-distilled water.

Extraction of protein from yeasts

Portions (4 ml) of yeast suspensions that had been prepared for fermentation rate determinations (p 59) were centrifuged in tapered glass centrifuge tubes (10 ml) at 2000 *g* for 10 min. The supernatants were discarded and each yeast pellet was resuspended in N-NaOH (2 ml). The tubes were covered with aluminium foil, placed in a bath of boiling water for 15 min, then centrifuged as before. The supernatant from each one was decanted into a volumetric flask (10 ml). The yeast residues were extracted twice more at 20°C with N-NaOH, and the combined extracts from each yeast strain were made up to 10 ml with N-NaOH.

Determination of protein in extracts

Portions (0.1 ml, 0.2 ml and 0.4 ml) of these diluted extracts were dispensed into test-tubes (16 mm x 15 cm), and the volume in each tube

was made up to 0.5 ml with glass-distilled water. The determination then followed the method of Lowry et al (1951). Standards ranging from 0 to 50 μg protein per tube were prepared using bovine serum albumin (Sigma Chemicals Co., Kingston-upon-Thames). Concentrations of protein in the extracts were calculated by reference to the standard curve.

Carbohydrate Determinations

All glassware used for these determinations was carefully cleaned with chromic acid, and rinsed with glass-distilled water that had been filtered through a cellulose-acetate membrane filter (47 mm diameter; pore size 0.22 μm; Millipore) to remove any dust particles which would interfere with the analysis. The glassware was then dried in an oven. Water used for dilution and preparation of standard and test solutions was glass-distilled, and filtered as described above.

Duplicate portions (5 ml) of suitable dilutions of each sugar solution under test, were dispensed into stoppered tubes (30 ml). Standard solutions were prepared, containing 10, 20 and 30 mg glucose per litre, and duplicate portions (5 ml) of these and of filtered glass-distilled water (to use as a blank) were also added to stoppered tubes. All tubes were placed in a mixture of methanol and solid carbon dioxide, and frozen. Anthrone reagent (10 ml; 0.2 g anthrone, recrystallized from ethanol, in 100 ml cold concentrated sulphuric acid) was added to each tube, and the contents of the tubes were refrozen. The tubes were then transferred to a water bath at 15°C and, after a short time, they were gently shaken to mix the solutions completely. At this time, all of the solutions were

a pale straw colour. The tubes were then heated at 90°C for 14 min, cooled rapidly to 15°C, and left to stand at room temperature for 15 min. The absorbance of each solution at 590 nm was measured against that of the blank. A standard curve was prepared and used to estimate the sugar contents of the test solutions.

Analyses on Worts and Beers

Beers produced by control and hybrid strains of yeast in canister (20 l) and on production-scale (60 brl, 168 brl and 480 brl) fermentations, together with the original worts, were examined using the following analyses.

Original gravity

These were measured using the method described by Savage (1971).

Present gravity

Specific gravities of the beers were measured using the specific gravity bottle method.

pH value

The beers were degassed by repeated transfers from one vessel to another, and the pH value was then measured using a pH meter (Vibron).

Colour

The colour of the beers was measured using the photometric method recommended by the Institute of Brewing (1971a).

Alcohol

The alcohol content of the beers was determined by the method in Statutory Instruments (1954).

Bitterness

The technique used was the method recommended by the Institute of Brewing (1971b).

Total nitrogen

The method used was a modification of that recommended by the Institute of Brewing (1971c). The modification involved using a portion of wort or beer (10 ml) for the analysis, and omitting the evaporation step.

Alpha-amino nitrogen

The amino acid contents of worts and beers were estimated using the trinitrobenzene-sulphonic-acid reagent technique, incorporating a modification of the method of Satake et al (1960). Phosphate buffer solution (5% w/v, disodium hydrogen orthophosphate in water, adjusted to pH 8.2 with aqueous sodium dihydrogen orthophosphate) was substituted for the sodium bicarbonate solution, and the volumes used were increased to 4 ml for the buffer solution, and 2 ml for the trinitrobenzene-sulphonic-acid reagent.

Total polyphenols

The method used estimates the total polyphenol content of worts and beers, and is a modification of the DeClerck and Jerumanis method (1967).

Diacetyl

Diacetyl contents of beers were estimated by the method of Owades and Jakovac (1963).

Dimethyl sulphide

The concentration of dimethyl sulphide in beers was estimated using the method of Sinclair et al (1969).

Volatile components

A conical flask (100 ml) containing sodium chloride (8 g) was flushed out with fresh air using a syringe (20 ml) and then sealed with a 'Suba-seal' stopper. A sample of beer (20 ml) and a portion (0.5 ml) of a standard solution of butan-1-ol (2 mg in one ml of 3.5% aqueous ethanol solution, v/v) were introduced into the flask through the Subaseal, using a syringe. The flask was then equilibrated in a water bath (30°C) for 30 min. A sample of vapour (10 ml) was taken with a gas syringe and injected on to a gas chromatograph column. The column was prepared using polyethylene glycol (20% w/w) on 'Embacel' (60-80 mesh) heated initially to 85°C, then increased to 130°C after 60 min. The carrier gas used was nitrogen at a flow rate of 100 ml/min, and the volatile compounds were detected using a flame ionisation detector (Philips PV 4000 or F & M Model 810).

The concentrations of volatile components in beers were calculated from peak heights. The following volatiles were estimated using this technique; acetaldehyde, 3 methyl-1-butanol acetate, butan-2-ol, ethyl acetate, 3-methyl-1-butanol together with 2-methyl-1-butanol, and

n-propanol. Total fusel oils were calculated from the sum of the concentrations of butan-2-ol, 3-methyl-1-butanol and 2-methyl-1-butanol.

PREPARATION OF MALTOTRIOSE

Maltotriose was obtained from Dr A MacPherson, Allied Breweries Process Research Department. This was contaminated with maltose (two to six per cent).

A sterile solution of the maltotriose (ca 2% w/v) was inoculated, at a level of 100×10^6 cells/ml, with a slurry of yeast strain Y 366. The slurry was prepared by growing the yeasts in MYGP broth overnight. The culture was centrifuged at 600 g under sterile conditions in an MSE Major centrifuge, and the cells were suspended in sterile glass-distilled water, and recentrifuged twice. After inoculation the solution of maltotriose was then incubated statically overnight at 25°C. Yeast Y 366 was unable to utilise maltotriose, but metabolised the maltose. After treatment, the solution was sterilised by filtration (membrane filter; 47 mm diameter; pore size 0.22 mμ; Millipore) and paper chromatography (Harris et al, 1951) was used to check whether all of the maltose had been removed. The actual concentration of maltotriose remaining in solution was determined using the anthrone technique (p 64). The solution was then diluted to 1.5% w/v.

CHEMICALS

All chemicals used were of the purest grade available obtained from Hopkin and Williams, Chadwell Heath, (Analytical Reagent) unless otherwise

stated. The sources of the chemicals and reagents used, are indicated where reference is first made in the script.

RESULTS

PROPERTIES OF PARENT STRAINS

The parent yeasts selected for this investigation include a range of strains of Saccharomyces cerevisiae and Saccharomyces carlsbergensis, some used in breweries, others in bakeries or distilleries. These strains are listed in the Materials and Methods section (Table 1, p 28). The properties of some of these strains in malt-wort fermentations are summarised and discussed in the section on Properties of Hybrid Strains (p 85).

Sporulating Ability

The ability of the parent strains to sporulate varies considerably ranging from an apparent inability (strains Y 86, Y 90, Y 95 and Y 311; Tables 3 and 4) to over 70% sporulation (strains Y 8, Y 26 and Y 261; Table 3). Strains of Sacch. carlsbergensis (Tables 4 and 5) produced spores more slowly than strains of Sacch. cerevisiae (Table 3), for evidence of sporulation only appeared after 72 h in the former. With the exception of Y 43, a non-brewing strain, Y 92 and Y 93, they sporulated poorly or not at all.

Mating Ability of Strains Derived from Parent Yeasts

When mating types of the non-sporing strains were examined, all of the parent strains produced some spore isolates that were sterile, ranging from 22% to 100% of the total (Tables 3, 4 and 5) and some yeasts produced strains capable of mating with either a or α mating strains.

TABLE 3

Sporulating ability and distribution of mating type among non-sporing isolates derived from parent strains of Saccharomyces cerevisiae

Strain number	Percent sporulation after		Percent of mating strains of mating type			Percent of sterile strains	Total number of isolates examined for mating type
	72 h	168 h	a	α	a/α		
Y 1 - 1	6.1	-	0	10.0	10.0	80.0	10
Y 1 - 2	(52.0)	-	38.2	3.6	0	58.2	55
Y 7	24.9	-	11.4	34.3	22.9	31.4	35
Y 8	82.1	-	31.2	37.5	6.3	25.0	16
Y 9	0	2.3	0	0	0	100	8
Y 10	17.8	-	44.4	11.1	22.2	22.2	9
Y 11	0	< 1	-	-	-	-	0
Y 26	(75.0)	-	16.7	15.3	5.5	62.5	72
Y 32	62.0	-	42.9	14.3	14.3	28.6	14
Y 33	30.9	-	0	50.0	0	50.0	2
Y 80	37.1	-	3.6	4.6	1.8	90.0	110
Y 86	(0)	(0)	-	-	-	-	0
Y 261	76.4	-	0	38.5	0	61.5	13
Y 304	35.8	-	23.5	5.9	2.0	68.6	51
Y 307	(56.0)	-	0	0	0	100	58
Y 311	(0)	(0)	-	-	-	-	0

- indicates that the strain was not tested

( ) indicates that a different batch of sporulation agar was used for these sporulation tests

TABLE 4

Sporulating ability and distribution of mating type among non-sporing isolates derived from parent strains of Saccharomyces carlsbergensis

Strain number	Percent sporulation after		Number of mating strains of mating type			Number of sterile strains	Total number of isolates examined for mating type
	72 h	168 h	a	α	a/α		
Y 41	0	5	0	0	0	2	2
Y 43	0	50	1	4	2	2	9
Y 90	0	0	-	-	-	-	0
Y 92	0	10	0	0	0	4	4
Y 93	0	20	0	0	1	5	6
Y 94	0	<1	0	0	0	1	1
Y 95	0	0	-	-	-	-	0
Y 140	0	<1	-	-	-	-	0

- indicates that the strain was not tested

TABLE 5

Sporulating ability of poorly sporulating parent strains of Saccharomyces cerevisiae and Saccharomyces carlsbergensis and the viability, sporulating ability and distribution of mating type among these strains when subjected to micromanipulation

Strain Number	Percent sporulation after 72 h	Percent sporulation after 168 h	Percent of viable strains obtained	Percent of viable strains capable of sporulation	Number of mating isolates of mating type a	α	a/α	Number of sterile isolates	Total number of isolates examined for mating type
Saccharomyces cerevisiae									
Y 9-1	0	34.8	77.8	63.8	21	0	0	0	21
Y 11	0	>1	0	0	-	-	-	-	0
Saccharomyces carlsbergensis									
Y 41	0	3	15.8	76.9	4	1	3	5	13
Y 93	0	10	93.1	31.4	5	4	5	21	35
Y 140	0	<1	83.3	82.8	2	7	2	22	33

- indicates that the strain was not tested

Y 9-1 is a recombinant obtained from parent yeast Y 9, which is capable of sporulating more readily than its original parent

The sporulating ability of strains Y 9, Y 11 and Y 140, which are thought to be useful parent types, was very low, and it had proved impossible to isolate fertile mating strains by the 'mass isolation' methods, employing plating techniques (Tables 3 and 4). Micromanipulation offered an alternative method of isolating the few asci produced, and of separating the spores. In addition, strains Y 41 and Y 93, which sporulated to a reasonable extent but which gave seven sterile cultures out of eight isolates using the 'mass isolation' methods, were subjected to the technique of micromanipulation.

This technique did not produce viable spores from Y 11 (Table 5) and spores of Y 9 within a two-spored ascus proved very difficult to separate from each other. However, one four-spored ascus was found in the sporulating culture of Y 9 during micromanipulation and this was isolated, grown up in wort and spore formation induced. This culture, designated Y 9-1, gave a higher percentage sporulation than the parent culture (ca 35%). Micromanipulation of a sporulating culture of this isolate produced 21 viable mating strains of the a mating type (Table 4) some of which could sporulate.

The sporulating cultures of strains Y 41, Y 93 and Y 140 were all successfully subjected to micromanipulation (Table 5) giving rise to mating strains. The α and a/α mating strains derived from Y 41 and Y 140 had poor mating ability with the standard strains STa and STα, and would not mate at all when hybridised with a and α strains from other parent yeasts (see Table 8, p 78). These yeasts, in practice behave as sterile strains, and perhaps should be included in this group giving 69.2 and 93.9% sterile strains, produced by Y 1 and Y 140 respectively.

Cell Volumes of Parent Yeasts and Mating Strains

Average cell volumes of parent and mating strains are listed in Table 6. Wort composition had a considerable effect on cell volume, and therefore wort batch numbers are quoted with each set of results.

MATING

All of the two hundred mating strains obtained from the parent strains were hybridised with one another as appropriate, with the following exceptions:-

1. Matings were not attempted between cultures derived from the same parent because it was not the policy of the hybridisation programme, for such inbreeding was thought to be unlikely to produce commercially useful hybrid strains.

2. Mating strains derived from Y 26 and Y 80 were not crossed with Y 8, Y 10, Y 32, Y 33 and Y 261 mating strains, and those from Y 9 were not hybridised with mating strains from Y 10 and Y 32 (Table 7). This is indicated by ND in the Tables. Also mating strains of Y 26 were not mated with those from Y 43 (Table 8). The six yeast strains Y 8, Y 10, Y 32, Y 33, Y 43 and Y 261 had not proved to be successful parent strains in earlier crosses, since they had failed to produce hybrids with useful brewing characteristics.

3. For the same reason, selected crosses only were made with mating strains derived from Y 41 and Y 140, using Y 1, Y 26 and Y 80 as the other parents (Table 8).

TABLE 6

Average cell volumes of parent strains of Saccharomyces cerevisiae and Saccharomyces carlsbergensis and mating and sterile strains derived from them

Strain number	Batch of wort used	Average volume of cell of parent strain (μm^3)	Average volume (μm^3) of cell of strain of mating type			
			a	α	a/α	Sterile
Saccharomyces cerevisiae						
Y 1 - 1	1	268.8			123.3	
	2	269.7		199.1		200.0
Y 7	1	187.5	80.0			
	3	191.1	91.1			124.4
Y 9	2	200.2				160.2
	4	242.8	79.4			192.3
			155.6			
Y 10	1	252.5		150.0		
	2	256.1	165.5		101.8	148.2
Y 26	5	252.4		131.2		
	6	210.3				
Y 32	5	163.7	94.8	58.9	178.9	90.6
Y 33	3	137.9		137.6		172.1
Y 80	6	347.1				
Y 261	7	118.7		50.1		49.7
Y 304	7	106.7	55.8			
	8	128.3				
Saccharomyces carlsbergensis						
Y 41	5	195.6				144.1
Y 92	8	65.5				70.0
Y 93	7	76.2				50.2
	3	147.1				97.0
Y 94	8	74.7				86.9
Y 95	9	221.0				
Y 140	9	219.7				
	9	228.8				

Each average value quoted is calculated from the average length and width obtained by measuring 100 randomly selected cells. The standard deviations of the calculated mean lengths and widths, expressed as a percentage of the average measurements lay between 11% and 24%, average around 17%

TABLE 7

Percentage compatibility of crosses between mating strains derived from parent strains of Saccharomyces cerevisiae

α and a/α mating strains from parent strain number	a and a/α mating strains from parent yeasts, with the strain numbers								
	Y 1	Y 7	Y 8	Y 9	Y 10	Y 26	Y 32	Y 80	Y 304
Y 1	ND	0	16.7	7.1	14.3	17.6	25.0	16.7	13.5
Y 7	0.7	ND	25.0	0	21.3	18.5	37.5	16.7	60.0
Y 8	0	0	ND	0.8	16.7	ND	35.4	ND	0
Y 10	0	16.7	44.4	ND	ND	ND	25.0	ND	0
Y 26	18.5	31.0	ND	18.7	ND	ND	ND	70.8	7.7
Y 32	33.3	50.0	62.5	ND	42.9	ND	ND	ND	75.0
Y 33	100	25.0	83.3	28.6	85.7	ND	87.5	ND	100
Y 80	40.3	6.0	ND	13.6	ND	51.3	ND	ND	15.4
Y 261	40.0	0	36.7	13.3	14.3	ND	35.0	ND	20.0
Y 304	33.0	0	8.3	13.1	ND	38.2	43.8	25.0	ND

ND indicates that no crosses were made between mating strains derived from these yeasts

TABLE 8

Percentage compatibility of crosses between mating strains derived from parent strains of Saccharomyces cerevisiae and Saccharomyces carlsbergensis

α and a/α mating strains derived from strains of Saccharomyces carlsbergensis strain number	a and a/α mating strains derived from strains of Saccharomyces cerevisiae, strain numbers							
	Y 1	Y 7	Y 8	Y 10	Y 26	Y 32	Y 80	Y 304
Y 41	0	ND	ND	ND	0	ND	0	ND
Y 43	66.7	75.0	91.7	47.6	ND	100	43.3	72.2
Y 93	0	100*	33.3*	28.6*	0	12.5*	0	0
Y 140	0	ND	ND	ND	0	ND	0	ND

a and a/α mating strains derived from strains of Saccharomyces carlsbergensis strain number	α and a/α mating strains derived from strains of Saccharomyces cerevisiae, strain numbers									
	Y 1	Y 7	Y 8	Y 10	Y 26	Y 32	Y 33	Y 80	Y 261	Y 304
Y 41	50.0	ND	ND	ND	64.3	ND	ND	57.1	ND	ND
Y 43	33.3	66.7	77.8	77.8	ND	50.0	100*	95.2	80.0	33.3
Y 93	60.0	0*	0*	0*	61.4	20.0	100*	72.9	0*	0*
Y 140	75.0	ND	ND	ND	35.7	ND	ND	57.1	ND	ND

ND indicates that no crosses were carried out between mating strains derived from these two yeasts

* indicates that only one mating strain of Y 33 and of Y 93 was available when these crosses were carried out

Mass isolation of spores proved unsuccessful for most strains of Sacch. carlsbergensis, because of their poor sporulating ability. However, it was found that strains Y 41, Y 92, Y 94, Y 95 and Y 140 would themselves mate with two very fertile mating strains, Y 8 a/α 1 and Y 33 α 1, derived from Sacch. cerevisiae strains Y 8 and Y 33 respectively. The hybrid strains produced sporulated more readily than their parent strains of Sacch. carlsbergensis, and could be used to produce additional hybrids. Mating strains could not be obtained from hybrid H 91, a cross between Y 94 and Y 33 α 1, and hybrid H 97 produced only a single sterile culture (Table 9). The others, H 94, H 95, H 96, H 98 and H 99, produced a range of fertile mating cultures (Table 9). These were crossed with selected mating cultures derived from strains of Sacch. cerevisiae and Sacch. carlsbergensis (Table 10). In addition, two other hybrid strains, H 10 and H 13, which are found to be fast fermenting yeasts, were induced to sporulate. Mating strains were isolated and crossed with maters from hybrid strains H 94, H 95, H 96, H 98 and H 99 and parent strains Y 10 and Y 304 (Table 11).

COMPATIBILITY

Calculated compatibilities for crosses between mating strains varied between 0 and 100% (Tables 7, 8, 10, 11). Averaged percentage compatibilities for crosses between different types of strains of Sacch. cerevisiae and Sacch. carlsbergensis are given in Table 12. The lowest average compatibility was 17.2%, among crosses between mating strains from brewing yeast (Sacch. cerevisiae). The highest average compatibility

TABLE 9

Distribution of mating type among non-sporulating isolates derived from selected hybrid strains

Hybrid number	Component mating strains of these hybrids		Number of mating strains of mating type			Number of sterile strains	Total number of non-sporing isolates examined for mating type
	a or a/α	x α or a/α	a	α	a/α		
H 94	Y 94	x 8 a/α 6	1	0	1	2	4
H 95	Y 95	x 33 α 1	1	1	1	1	4
H 96	Y 41	x 33 α 1	1	3	2	4	10
H 97	Y 140	x 33 α 1	0	0	0	1	1
H 98	Y 92	x 33 α 1	0	2	1	0	3
H 99	Y 41	x 8 a/α 6	0	2	0	1	3
H 10	7 a 4	x 10 α 4	1	4	0	12	17
H 13	8 a/α 6	x 1 α 1	4	3	7	4	18

TABLE 10

Percentage compatibility of crosses between mating strains derived from selected hybrid yeasts with those derived from parent strains of Saccharomyces cerevisiae and Saccharomyces carlsbergensis

α and a/α mating strains from hybrid strains	a and a/α mating strains derived from parent yeasts with the strain numbers							
	Y 1	Y 7	Y 8	Y 10	Y 32	Y 43	Y 93	Y 304
H 94	100	50.0	66.7	57.1	25.0	0	0	0
H 95	50.0	0	33.3	0	12.5	33.3	0	16.7
H 96	40.0	40.0	50.8	34.3	52.5	86.7	20.0	46.7
H 98	33.3	66.7	66.7	61.9	41.7	66.7	33.3	33.3
H 99	0	25.0	25.0	7.1	43.8	66.7	50.0	0

a and a/α mating strains from hybrid strains	α and a/α mating strains derived from parent yeasts with the strain numbers									
	Y 1	Y 7	Y 8	Y 10	Y 32	Y 33	Y 43	Y 93	Y 261	Y 304
H 94	25.0	50.0	33.3	16.7	87.5	100	90.0	50.0	50.0	25.0
H 95	0	0	0	0	0	50.0	0	50.0	0	0
H 96	50.0	33.3	27.7	11.1	50.0	100	55.5	33.3	60.0	33.3
H 98	50.0	0	0	0	50.0	100	50.0	0	100	50.0

Numbers of mating strains involved in crosses are given in Tables 3, 4, 5 and 9

TABLE 11

Percentage compatibility of crosses between mating strains derived from hybrid strains H 10 and H 13 with those derived from hybrid strains H 94, H 95, H 96, H 98 and H 99 and from parent strains of Saccharomyces cerevisiae, Y 10 and Y 304

a and a/α mating strains derived from hybrid strains	α and a/α mating strains derived from yeast strains						
	H 94	H 95	H 96	H 98	H 99	Y 10	Y 304
H 10	100	0	20.0	33.0	0	0	100
H 13	54.5	27.3	31.0	27.3	22.7	0	63.6

α and a/α mating strains derived from hybrid strains	a and a/α mating strains derived from yeast strains					
	H 94	H 95	H 96	H 98	Y 10	Y 304
H 10	50.0	83.3	44.4	33.3	42.9	0
H 13	25.0	70.0	30.0	0	20.0	23.3

Numbers of mating strains involved in crosses are given in Table 9

TABLE 12

Average percent compatibilities for crosses between mating strains derived from brewing and non-brewing strains of Saccharomyces cerevisiae and Saccharomyces carlsbergensis and hybrid strains H 94, H 95, H 96, H 98 and H 99 and H 10 and H 13

	Average percent compatibility				
	Strains of Saccharomyces cerevisiae		Strains of Saccharomyces carlsbergensis		Hybrid strains H 10 and H 13
	Brewing	Baking/ distilling	Brewing	Non-brewing	
Brewing strains of Saccharomyces cerevisiae	17.2	44.4	26.7 (34.8)	65.1	31.1
Baking or distillery strains of Saccharomyces cerevisiae	44.4	61.3	44.2	82.5	ND
Hybrid strains H 94, H 95, H 96, H 98 and H 99	32.8	62.4	26.3	56.1	31.2

ND indicates that no crosses were carried out between mating strains derived from these parent strains

was 82.5%, obtained when baking and distillery strains of Sacch. cerevisiae were hybridised with a non-brewing strain of Sacch. carlsbergensis. Overall, non-brewing strains produced mating strains that were more fertile than those derived from brewing 'ale' and 'lager' yeasts.

Although crosses between brewing strains of Sacch. cerevisiae and Sacch. carlsbergensis were only 26.7% successful, it must be noted that the α and a/α mating strains of Y 41 and Y 140 would not mate with any of the parent strains. If these crosses are ignored, the compatibility rises to 34.8% (Table 12).

Strains H 94, H 95, H 96, H 98 and H 99 cannot be described as true hybrids, for they were produced by hybridising parent strains of Sacch. carlsbergensis with mating strains of Sacch. cerevisiae. The fertility of the mating strains derived from these hybrids was similar to that of brewing strains, but not as high as the fertility of the mating strains from true hybrids, such as baking and distillery yeasts and the non-brewing parent strain of Sacch. carlsbergensis. The a and a/α strains of H 95 mated very poorly in this experiment and the compatibility results for these crosses have not been included in the calculations. Also, only a single a mating strain of Y 1, one α strain of Y 33 and one a/α strain of Y 93, H 94 and H 98 were available for matings when these crosses were carried out, (Table 10).

PROPERTIES OF HYBRID STRAINS

Hybrid yeasts were examined for genetic stability after incubating on Wallerstein Laboratories Nutrient agar, and all stable strains, with less than 15 per cent abnormal colonies, were screened for fermentation behaviour using test-tube cultures. The genetic stability of parent strains of yeast and some of the hybrids are shown in Table 13. Selected strains were then examined in greater detail.

Characteristics of Hybrids in Test-Tube Cultures

The test-tube culture technique was designed to select, from the large number of organisms produced, hybrids with good fermentation characteristics. The yeasts were tested for flocculence, head formation, ability to attenuate wort, and duration of lag phase. Measurement of the first two properties is somewhat arbitrary and it is difficult to assess their reproducibility. However the classification groupings are wide (see key, Appendix I). The reproducibilities of the other two characteristics, namely attenuative ability and duration of lag phase, are also given at the front of Appendix I. Results of test-tube wort cultures are summarised in Table 14 and Appendix I.

Flocculence

A large proportion of hybrids (84.9 per cent, Table 14) had flocculence characteristics the same as, or intermediate between those of their parent strains. A small percentage (2.0) were more flocculent than either parent yeast, and 13.1 per cent of hybrids showed less flocculence. A large proportion (90.1 per cent) of the hybrids derived from crosses with

TABLE 13

Colonial morphology and estimated percentage abnormal colonies for parent strains and hybrid strains H 10 and H 13

Strain number	Description of normal colonial morphology after 5 days on Wallerstein Laboratory Nutrient Agar	Percent abnormal colonies
Strains of Saccharomyces cerevisiae		
Y 1	Green, rough, crenulated	4.5
Y 7	Green, rough, crenulated	0
Y 8	Green, smooth, entire	0
Y 9	Green, smooth, slightly crenulated	2
Y 10	Green, very rough, crenulated	0
Y 32	Dark green, smooth, slightly crenulated	0
Y 33	Dark green, smooth, entire	1
Y 80	Green, smooth, slightly crenulated	9
Y 86	Green, smooth, crenulated	1
Y 261	Dark green, smooth, entire	10
Y 304	Green, mixed rough and smooth, all crenulated	15
Strains of Saccharomyces carlsbergensis		
Y 41	Creamy, smooth, entire	0
Y 43	Pale green, smooth, entire	0
Y 92	Pale green, smooth, entire	2
Y 93	Pale green, smooth, crenulated	0
Y 94	Green, smooth, entire	0
Hybrid strains of Saccharomyces cerevisiae		
H 10	Green, raised centre, crenulated	1
H 13	Dark green, smooth, slightly crenulated	9

The percent abnormal colonies was calculated from the mean value for triplicate plates, each with *ca* 200 colonies

TABLE 14

Summary of the comparison of the fermentation characteristics of hybrid strains and their component parent strains

Fermentation characteristic	Percent of hybrids which, when compared with their parent strains have:-		
	Less flocculence.	Intermediate flocculence.	More flocculence.
Flocculence	13.1	84.9	2.0
	Weaker head forming ability.	Intermediate head forming ability.	Stronger head forming ability.
Head formation	6.2	90.2	3.6
	Less attenuative ability.	Intermediate attenuative ability.	More attenuative ability.
Attenuation	18.9	76.3	4.8
	Shorter lag phase.	Intermediate lag phase.	Longer lag phase.
Lag phase	1.8	80.2	18.0

The total number of hybrids is ca 2000

mating strains derived from yeast strain Y 9 were non-flocculent, and the only flocculent hybrids produced from this yeast were products of hybridisation with Y 26, a strongly flocculent strain. (Appendix 1)

When mating strains from hybrids H 94, H 95, H 96, H 98 and H 99 were crossed with those from parent strains (Table 15), 92.9 per cent of the offspring were non-flocculent and 3.55 per cent each of group II and group III flocculence. Genetically stable flocculent hybrids, which numbered fourteen out of a total of eighteen, were examined in test-tube cultures for their other fermentation characteristics.

Head formation

Most hybrids have head-forming characteristics the same as, or intermediate between, those of the parent strains (90.2%, Table 14). A small proportion have head-forming abilities which are stronger (3.6%) or weaker (6.2%) than either parent.

Of the hybrids formed between mating strains from the hybrid yeasts H 94, H 95, H 96, H 98 and H 99, and those from the parent strains, 14.3% were head-formers.

Attenuating ability

Again, the majority of hybrids formed between parent strains show attenuating ability similar to, or intermediate between, that of their parent strains, but 18.9% show poorer attenuation than either parent, and 4.8% show better attenuation capacity than either (Table 14).

When hybrids formed from mater cultures derived from H 94, H 95, H 96, H 98 and H 99 and those from parent strains were tested, 57.1% had poor attenuation, and 42.9% average attenuation.

TABLE 15

Distribution of flocculence among hybrids derived from crosses between mating strains of H 94, H 95, H 96, H 98 and H 99, with those from selected parent strains

Hybrid numbers	Flocculence type of yeast hybrid itself	Numbers of hybrids of each flocculence type produced from crosses with parent yeasts										Component parent strains of hybrids H 94 - H 99	
		Y 1 III	Y 7 II-III	Y 8 III	Y 10 II-III	Y 32 I-II	Y 33 I-II	Y 43 I	Y 93 I-II	Y 261 I	Y 304 II	Strain	Flocculence class
H 94	I	2 I	3 I	8 I	5 I	9 I	2 I	8 I	1 I	4 I	1 I	Y 94	I-II
										1 II		Y 8	III
H 95	I	1 II	-	2 I	-	2 I	1 I	2 I	1 I	-	1 I	Y 95	I
				2 III								Y 33	I-II
H 96	I	5 I	5 I	22 I	11 I	24 I	3 I	23 I	2 I	8 I	8 I	Y 41	II-III
			1 III		2 III	1 II				1 II		Y 33	I-II
						1 III							
H 98	I	2 I	4 I	12 I	13 I	10 I	1 I	9 I	1 I	5 I	4 I	Y 92	I-II
						2 II						Y 33	I-II
H 99	I	-	1 III	3 I	1 II	4 I	-	4 I	1 III	-	-	Y 41	II-III
						1 II						Y 8	III
						1 III							

- indicates that no hybrids were produced from these crosses.
I, II or III under parent strain numbers (eg Y 1) denote the flocculence type to which these yeasts belong.
The total number of hybrids was 252.

Lag phase

A large proportion of hybrids (80.2 per cent) had intermediate lag phases, and 18 per cent longer ones than either parent yeast.

Examples of results for test-tube cultures of selected hybrids and parent strains are given in Table 16.

Characteristics in Tube Cultures (1.5 litres)

Hybrid strains H 10, H 13, H 1431, H 1465 and H 1985 were all examined in 2-litre tubes. The results of these experiments are given in Figures 4 to 10 and Table 17. All five hybrids attenuated better or more readily than the strains with which they were compared (Figures 4, 6, 8, 9, 10) and excepting H 10, they produced lower yields of yeast, in wet weight (Table 17). The final yeast counts varied widely, hybrids H 10 and H 13 giving beers with high residual counts, H 1431 and H 1465 producing beers with lower counts than the control strain Y 1, and the beer from strain H 1985 having a similar cell count to its control (Table 17).

Hybrid strains H 10 and H 1465 produced pronounced heads (Figures 5 and 9) throughout the fermentations, whereas strains H 13 and H 1431 produced foam early in fermentation but this collapsed later (Figures 7 and 8). Strain H 1985 produced a head which was not as dense or as marked as that produced by control strain Y 1 (Figure 10).

In 36 tube fermentations (1.5 litre), each using a different batch of wort, yeast strain Y 1 produced beers with a mean specific gravity of 1.0133 (standard deviation 0.002), 90 h after inoculation.

TABLE 16

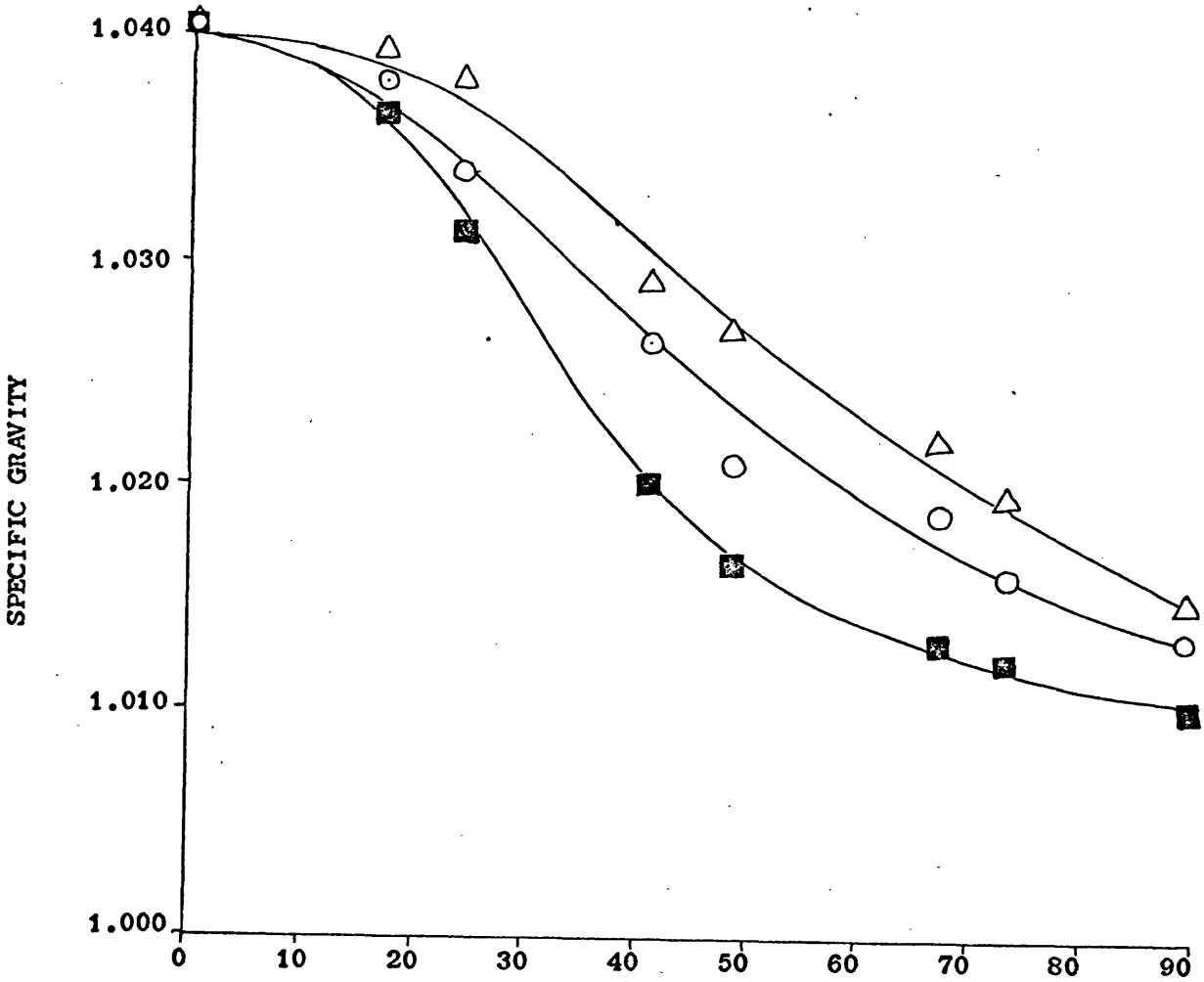
Properties of selected hybrid strains and control strains Y 1 and Y 80 in test-tube cultures

Yeast strain	Component mating strains of hybrids			Absorbancy in EEL units at 24 h	Specific gravity at 96 h	Flocculence	Head formation
	a or a/α	x	α or a/α				
H 10	Y 7 a 4	x	Y 10 α 4	2.2	1.011	I	+
H 13	Y 8 a 6	x	Y 1 a/α 1	3.9	1.0145	I	-
				2.1	1.014	III	++
H 1431	Y 26 a 22	x	Y 7 α 2	2.3	1.0115	IV	-
H 1465	Y 1 a 40	x	Y 26 α 23	2.0	1.012	IV	+
Y 1				2.1	1.0135	III	++
Y 80				2.6	1.016	IV	-
H 1985	Y 26 a 48	x	Y 7 α 19	2.8	1.011	II	++
H 1431	Y 26 a 22	x	Y 7 α 2	3.0	1.0105	IV	-
Y 1				3.0	1.0115	III	++
Y 80				3.2	1.0135	IV	-

The test-tube cultures were carried out in triplicate. Mean values are given for absorbancy at 24 h and specific gravity

FIGURE 4

CHANGES IN SPECIFIC GRAVITY DURING FERMENTATION OF TUBE CULTURES OF HYBRID STRAIN H 10 AND ITS PARENT STRAINS Y 7 AND Y 10



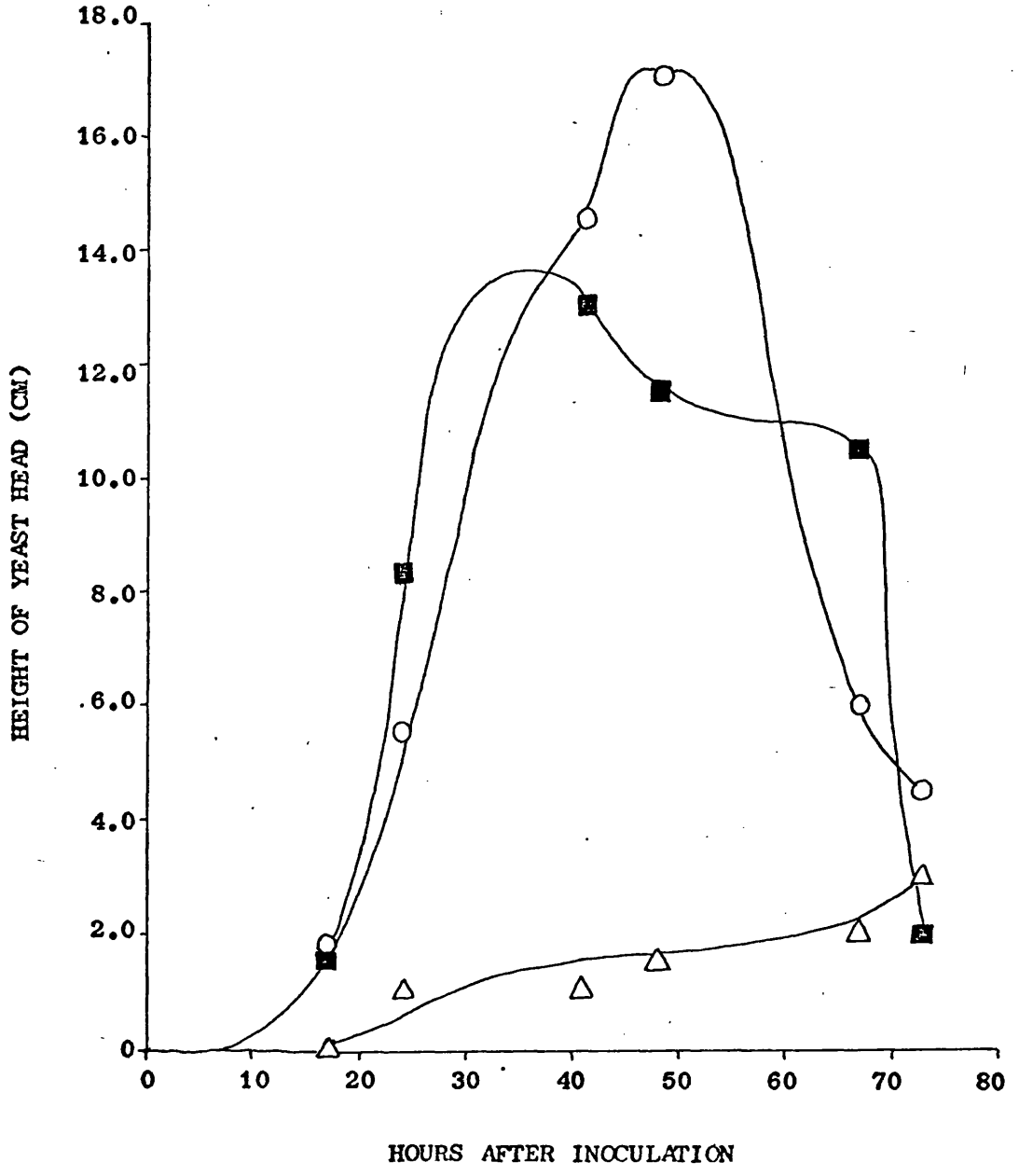
○ indicates behaviour of strain Y 7.

△ indicates behaviour of strain Y 10.

■ indicates behaviour of strain H 10.

FIGURE 5

CHANGES IN THE HEIGHT OF THE YEAST HEAD DURING FERMENTATION OF TUBE CULTURES OF HYBRID STRAIN H 10 AND ITS PARENT STRAINS Y 7 AND Y 10



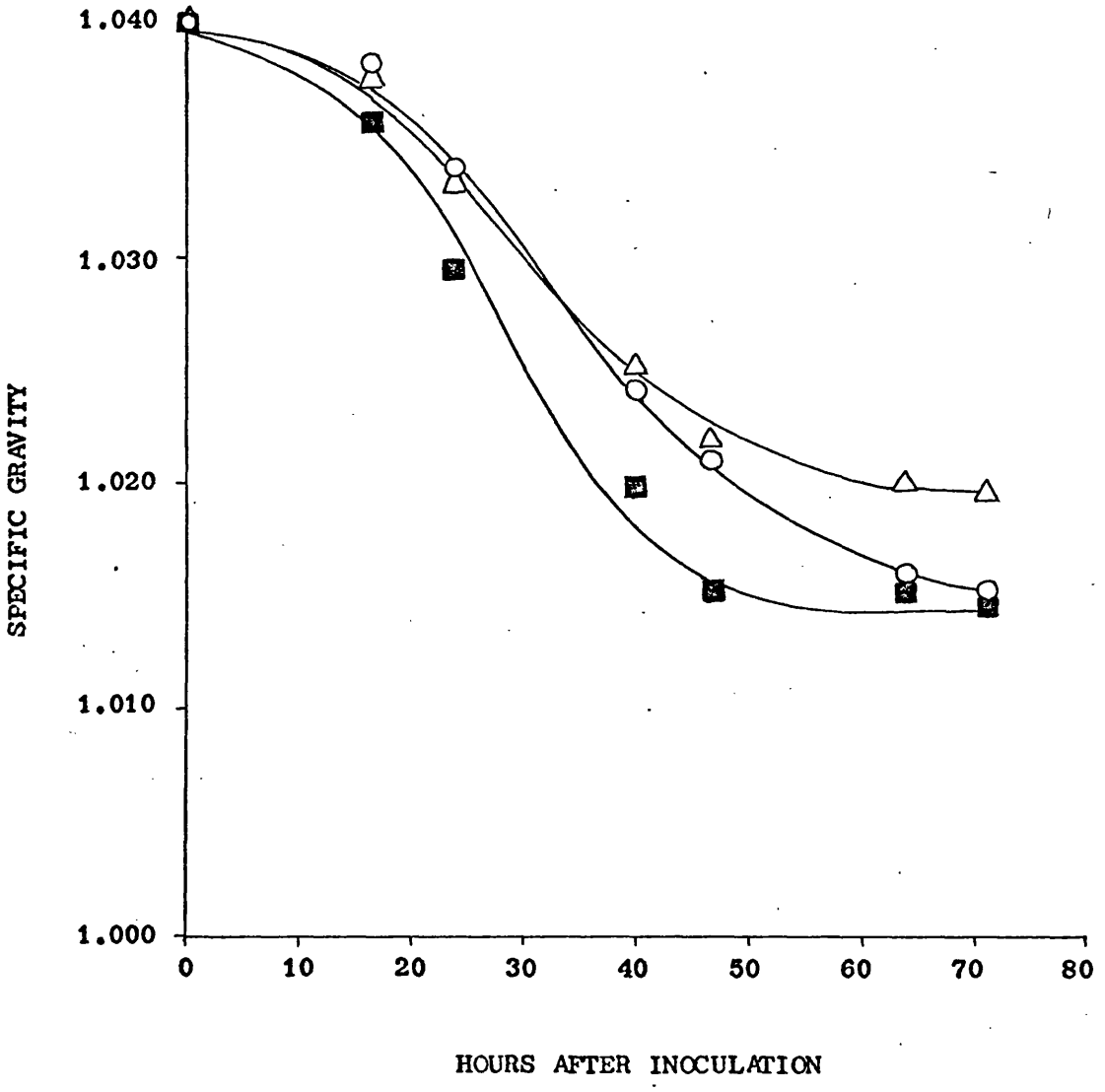
○ indicates behaviour of strain Y 7

△ indicates behaviour of strain Y 10

■ indicates behaviour of strain H 10

FIGURE 6

CHANGES IN SPECIFIC GRAVITY DURING FERMENTATION OF TUBE CULTURES OF HYBRID STRAIN H 13 AND ITS PARENT STRAINS Y 1 AND Y 8



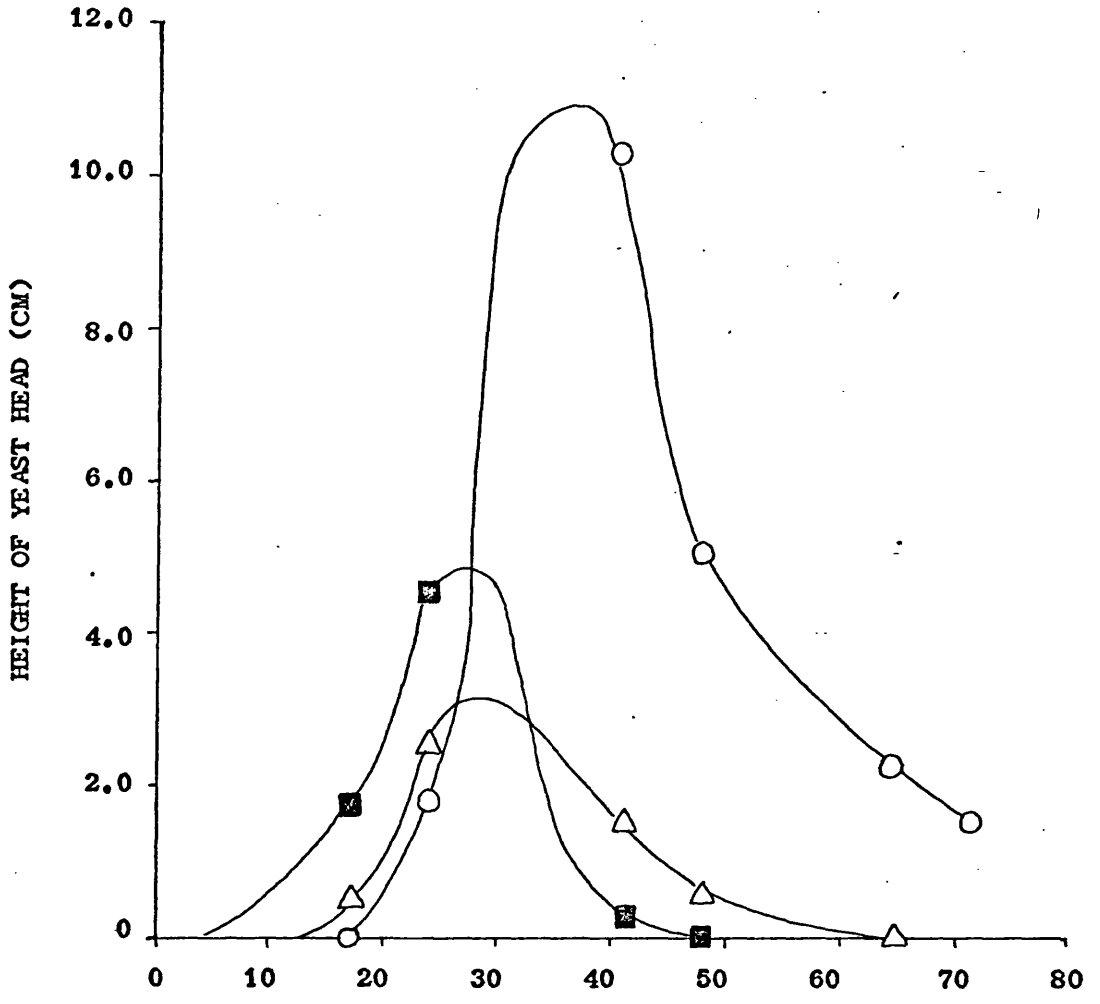
○ indicates behaviour of strain Y 1

△ indicates behaviour of strain Y 8

■ indicates behaviour of strain H 13

FIGURE 7

CHANGES IN THE HEIGHT OF THE YEAST HEAD DURING FERMENTATION OF TUBE CULTURES OF HYBRID STRAIN H 13 AND ITS PARENT STRAINS Y 1 AND Y 8



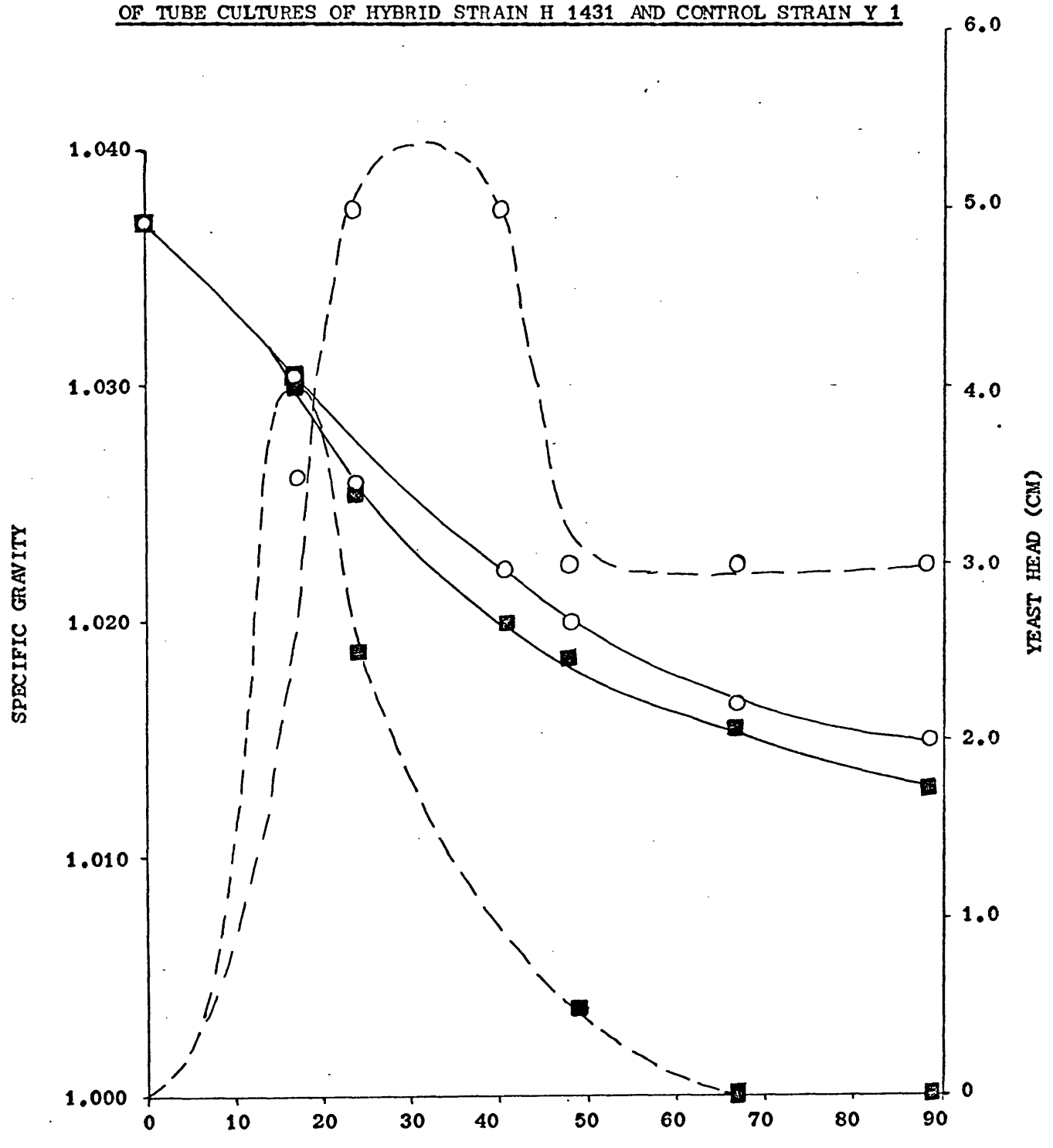
O indicates behaviour of strain Y 1

△ indicates behaviour of strain Y 8

■ indicates behaviour of strain H 13

FIGURE 8

CHANGES IN SPECIFIC GRAVITY AND THE HEIGHT OF THE YEAST HEAD DURING FERMENTATION OF TUBE CULTURES OF HYBRID STRAIN H 1431 AND CONTROL STRAIN Y 1



O indicates behaviour of strain Y 1

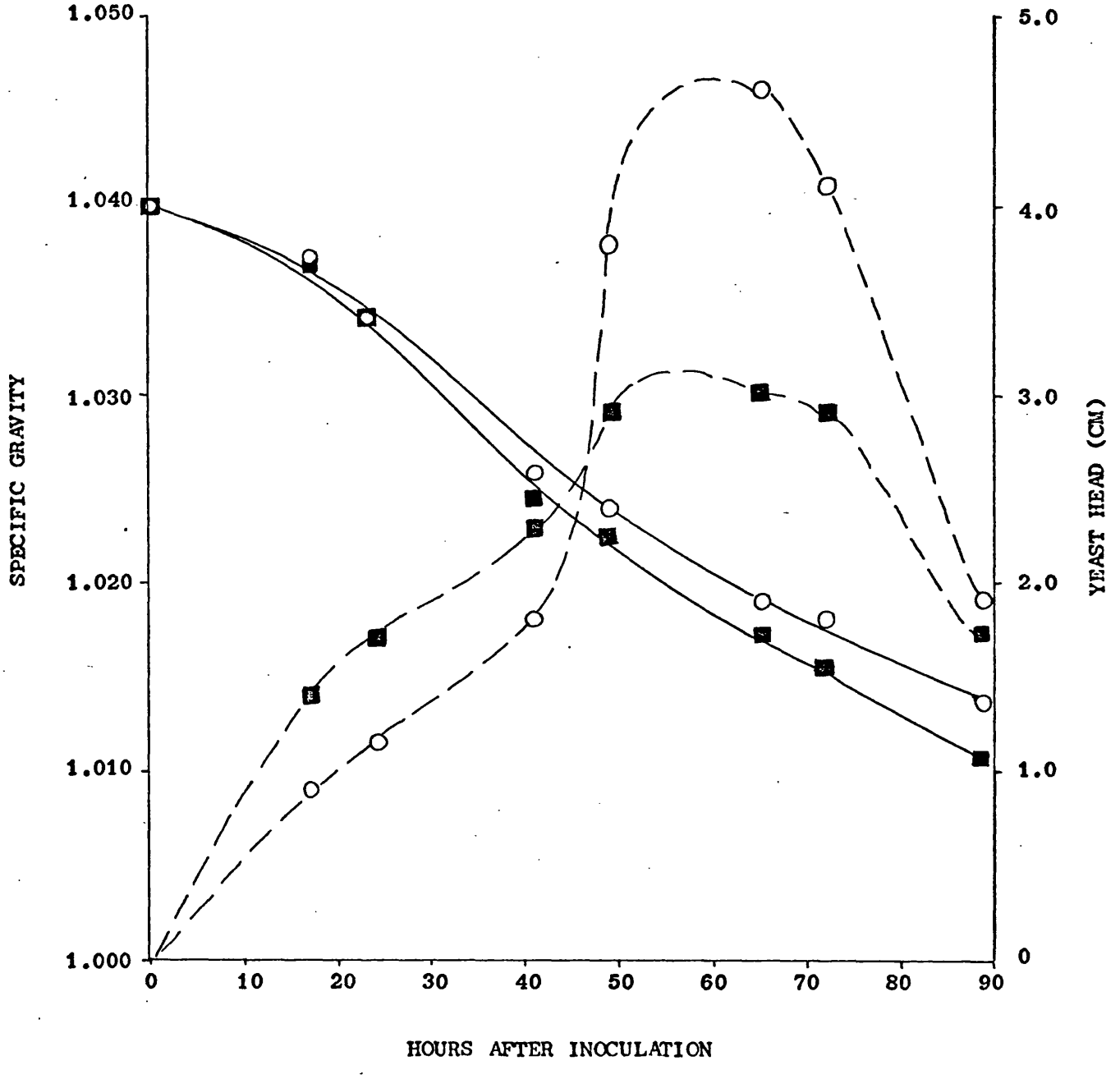
■ indicates behaviour of strain H 1431

——— indicates changes in specific gravity

– – – indicates changes in the height of the yeast head

FIGURE 9

CHANGES IN SPECIFIC GRAVITY AND THE HEIGHT OF THE YEAST HEAD DURING FERMENTATION OF TUBE CULTURES OF HYBRID STRAIN H 1465 AND CONTROL STRAIN Y 1



○ indicates behaviour of strain Y 1

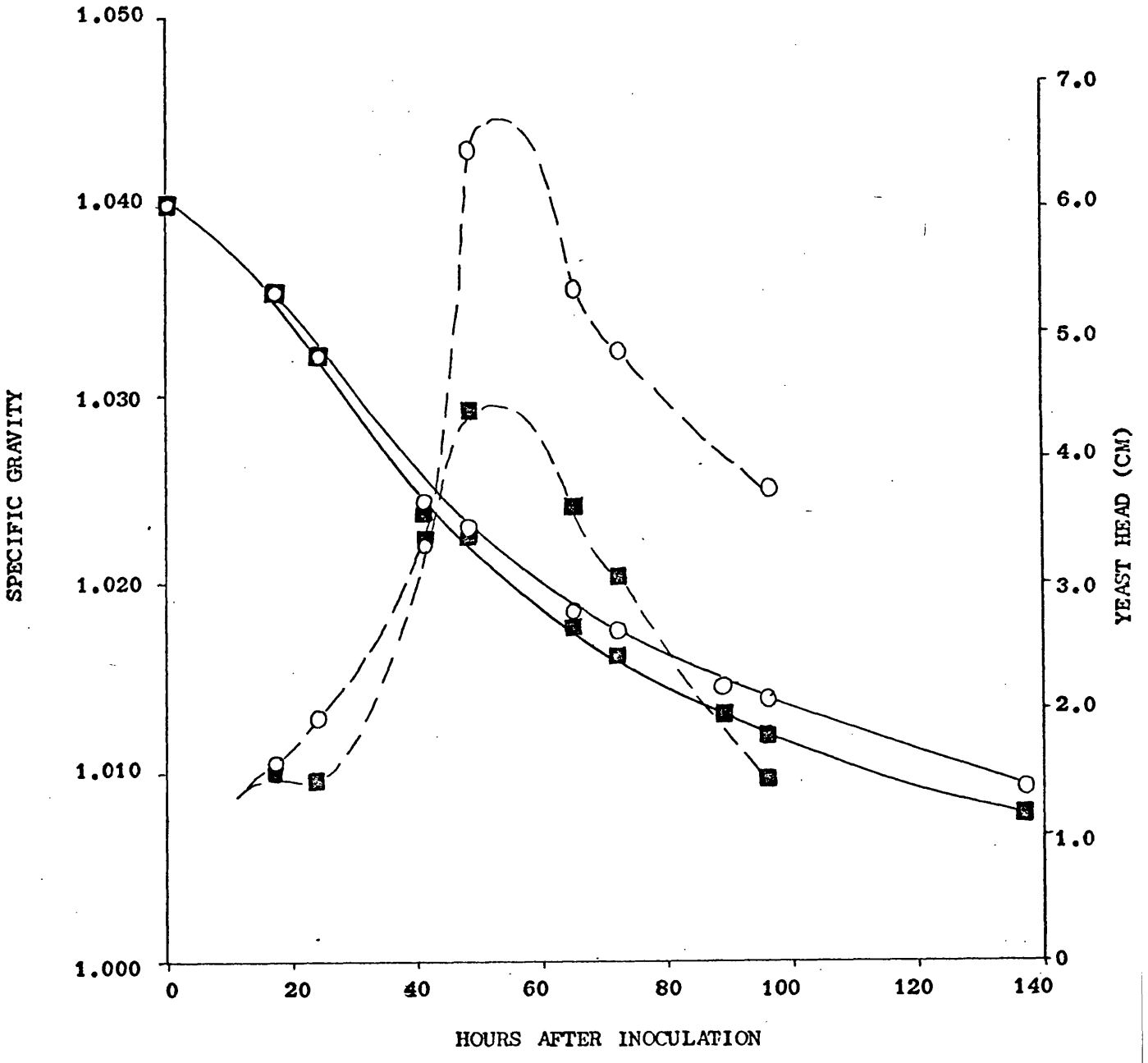
■ indicates behaviour of strain H 1465

——— indicates changes in specific gravity

– – – indicates changes in the height of the yeast head

FIGURE 10

CHANGES IN SPECIFIC GRAVITY AND THE HEIGHT OF THE YEAST HEAD IN FERMENTATIONS OF TUBE CULTURES OF HYBRID STRAIN H 1985 AND CONTROL STRAIN Y 1



○ indicates behaviour of Y 1

■ indicates behaviour of H 1985

——— indicates changes in specific gravity

— — — indicates changes in the height of the yeast head

TABLE 17

Specific gravities, yeast counts and wet weight yields at completion of tube cultures of hybrid and parent strains of yeast

Yeast strain	Component parent strains	Final specific gravity	Final yeast count (in millions per ml)	Yield of yeast (g wet weight) per litre
H 10	Y 7 x Y 10	1.010	33.2	21.0
Y 7		1.0135	12.5	18.5
Y 10		1.015	24.8	17.3
H 13	Y 1 x Y 8	1.0145	64.3	18.0
Y 1		1.0155	28.2	19.7
Y 8		1.0195	10.0	19.3
H 1431	Y 7 x Y 26	1.013	1.6	21.5
Y 1		1.014	8.1	25.9
H 1465	Y 1 x Y 26	1.0105	24.5	ND
Y 1		1.0135	35.0	ND
H 1985	Y 7 x Y 26	1.008	10.5	30.0
Y 1		1.0095	13.4	32.9

ND indicates that this was not measured.

Completion of tube fermentations was considered to be when the fermentations had a specific gravity below 1.012, or when the rate of fall of specific gravity was less than 1.001 in 24 hours. This was normally between 96 and 144 h after pitching.

Characteristics in Canister Cultures (20 litres)

Hybrid strains H 10, H 13, H 1431, H 1465 and H 1985 have all been examined in 20 l wort fermentations. The results are given in Figures 11-14. Yeast H 10, H 1431 and H 1465 all attenuated better than the control strain. Strains H 10 and H 13 produced beers with high residual counts (over 30 million cells per ml), whereas the beers produced by the two flocculent hybrids, H 1431 and H 1465, contained low concentrations of yeast cells of less than five million cells per ml.

Cell Sizes of Selected Hybrid Strains, Parent Yeasts and Component Mating Strains

The cell volumes of H 10, H 13, H 1431 and H 1465, their parent yeast strains and component mating types are given in Table 18.

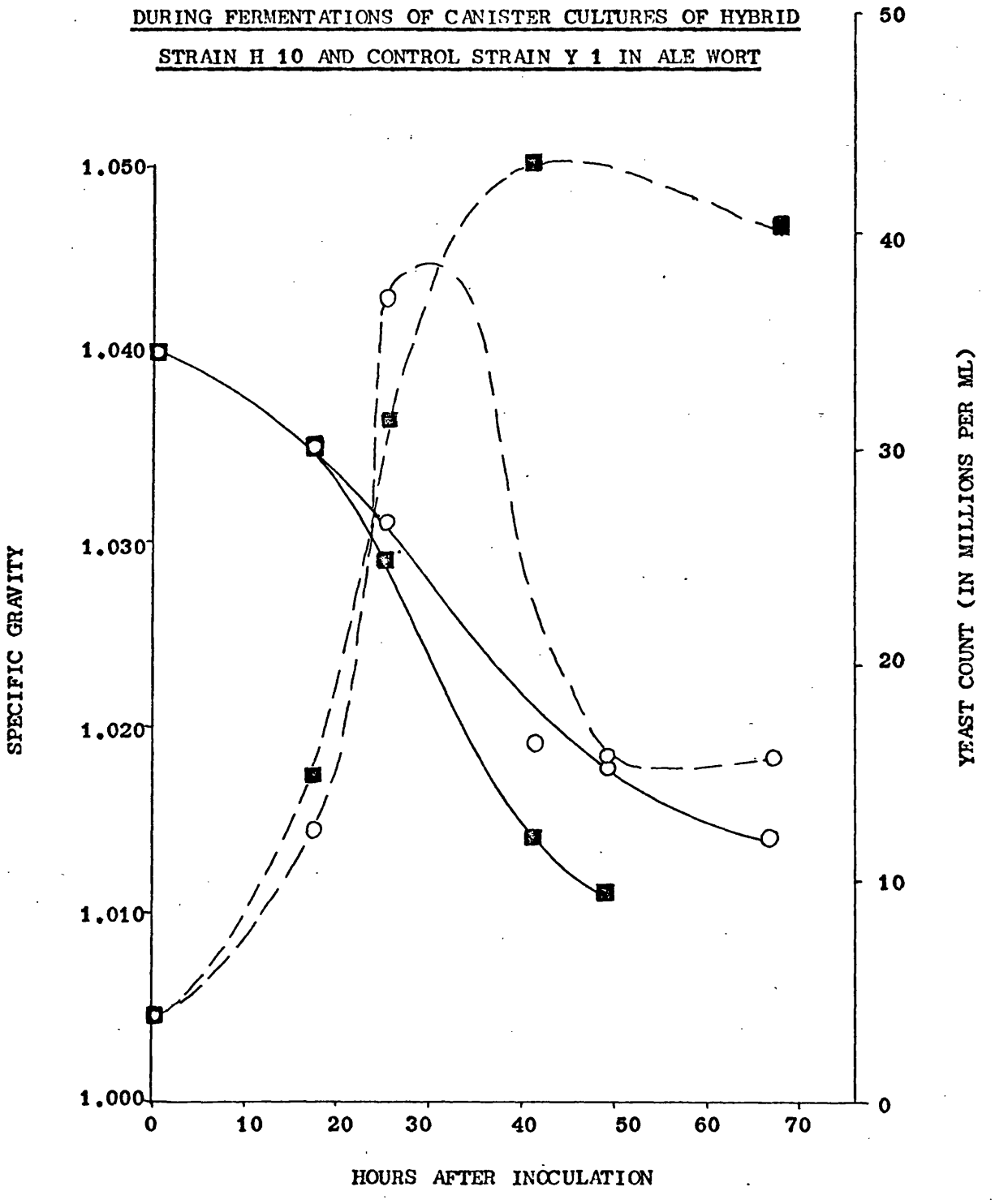
Specific Growth Rates and Duration of Lag Phases of Growth in Selected Yeast Strains

Examples of plots used to derive growth rates, generation times and the duration of lag phases are given in Figure 15, and specific growth rates and generation times for selected yeasts are quoted in Table 19.

The lag phases of growth in cultures of hybrid strain H 13 and its parent yeasts Y 1 and Y 8 can be deduced from Figure 16 as less than 15 h, approximately 16 h and 21 to 22 hours respectively.

FIGURE 11

CHANGES IN SPECIFIC GRAVITY AND THE COUNT OF YEAST CELLS IN SUSPENSION DURING FERMENTATIONS OF CANISTER CULTURES OF HYBRID STRAIN H 10 AND CONTROL STRAIN Y 1 IN ALE WORT



○ indicates behaviour of strain Y 1

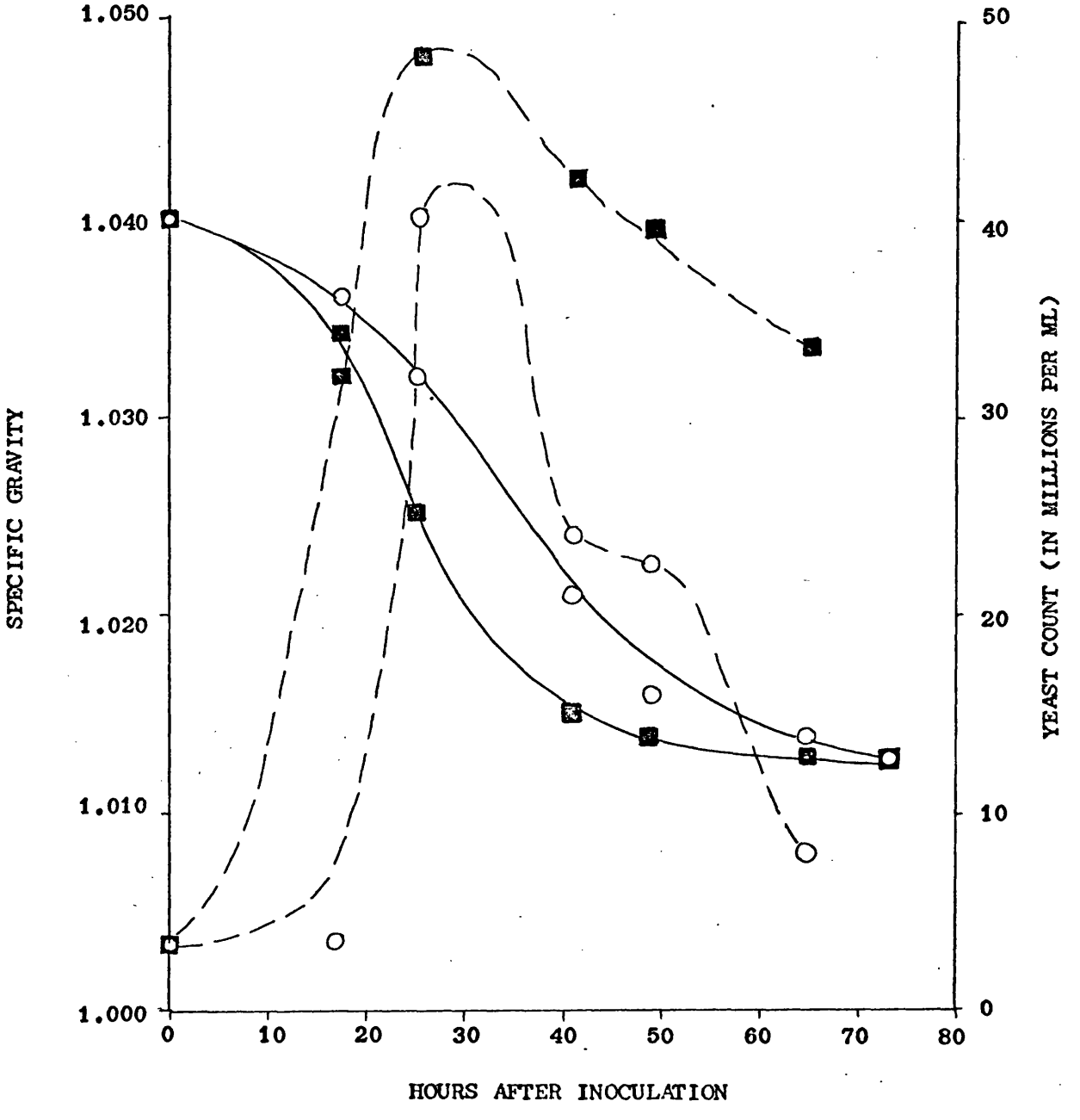
■ indicates behaviour of strain H 10

——— indicates changes in specific gravity

— — — indicates changes in the count of yeast cells

FIGURE 12

CHANGES IN SPECIFIC GRAVITY AND THE COUNT OF YEAST CELLS IN SUSPENSION DURING FERMENTATIONS OF CANISTER CULTURES OF HYBRID STRAIN H 13 AND CONTROL STRAIN Y 1 IN ALE WORT



○ indicates behaviour of strain Y 1

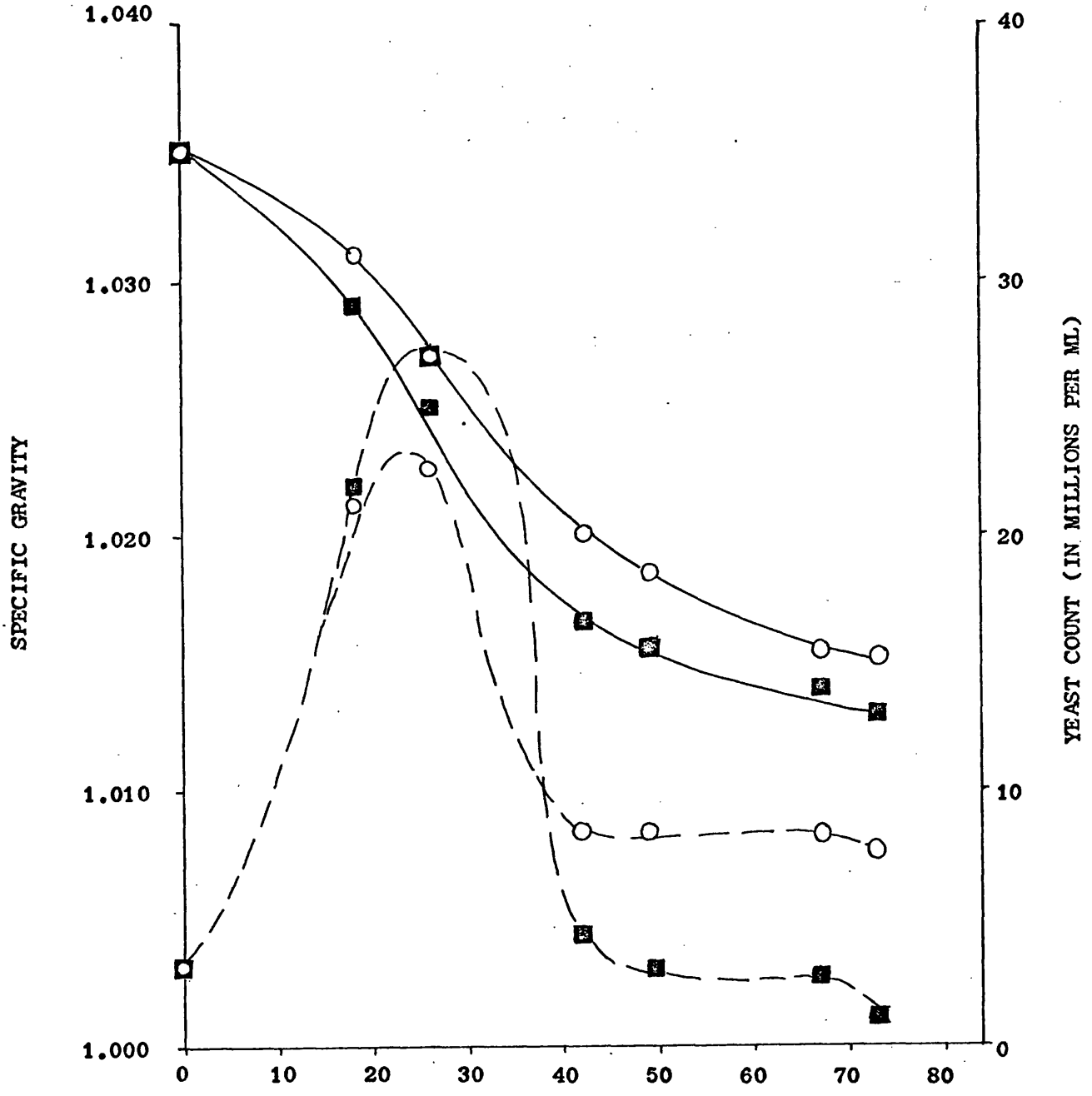
■ indicates behaviour of strain H 13

——— indicates changes in specific gravity

— — — indicates changes in the count of yeast cells

FIGURE 13

CHANGES IN SPECIFIC GRAVITY AND THE COUNT OF YEAST CELLS IN SUSPENSION DURING FERMENTATIONS OF CANISTER CULTURES OF HYBRID STRAIN H 1431 AND CONTROL STRAIN Y 1 IN ALE WORT



○ indicates behaviour of strain Y 1

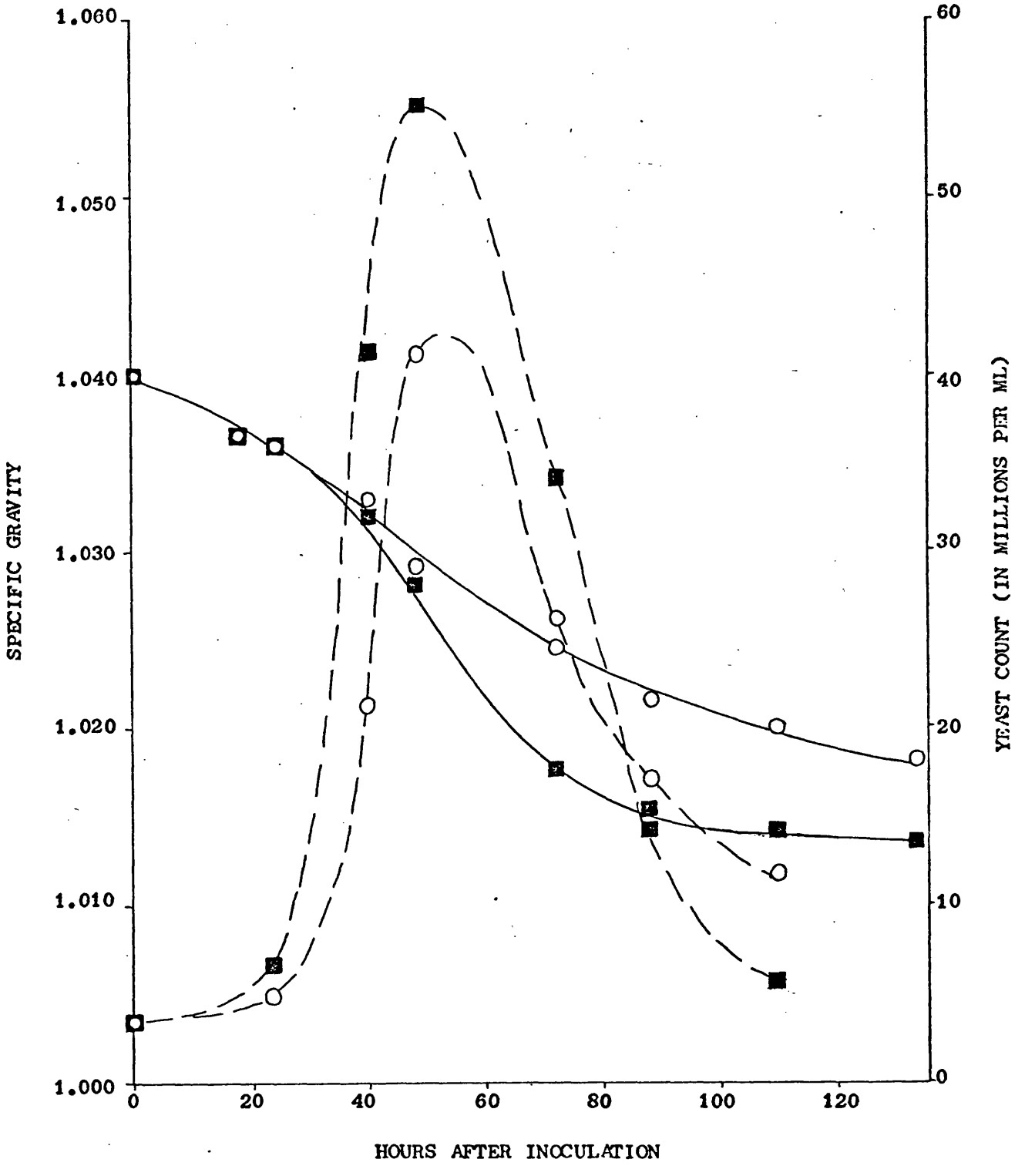
■ indicates behaviour of strain H 1431

——— indicates changes in specific gravity

— — — indicates changes in the count of yeast cells

FIGURE 14

CHANGES IN SPECIFIC GRAVITY AND THE COUNT OF YEAST CELLS IN SUSPENSION DURING FERMENTATIONS OF CANISTER CULTURES OF HYBRID STRAIN H 1465 AND CONTROL STRAIN Y 1 IN ALE WORT



○ indicates behaviour of strain Y 1

■ indicates behaviour of strain H 1465

——— indicates changes in specific gravity

— — — indicates changes in the count of yeast cells

TABLE 18

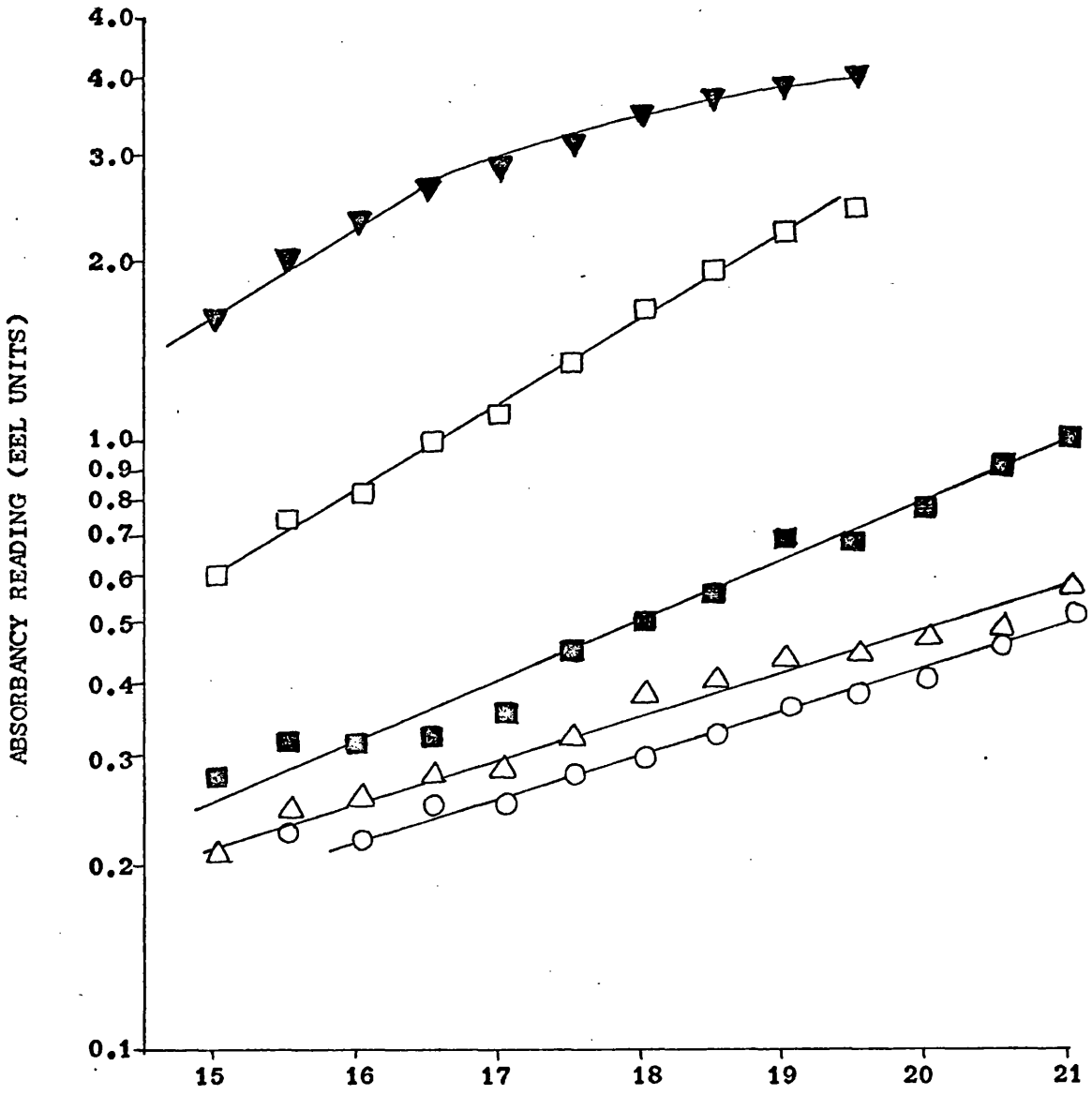
Average cell volumes of hybrids, parent strains and component mating strains of yeast

Yeast	Average cell volume in μm^3	Possible ploidy
H 10	157.5	diploid
Y 7	187.5	diploid or aneuploid
Y 10	252.5	triploid
Y 7 a 4	80.0	haploid
Y 10 α 4	150.0	diploid or aneuploid
H 13	244.0	triploid
Y 1	268.8	triploid
Y 8	273.3	triploid
Y 1 a/α 1	123.3	haploid or aneuploid
Y 8 a 6	193.8	diploid
H 1431	275.5	triploid
Y 7	191.1	diploid or aneuploid
Y 26	210.3	triploid
Y 7 α 2	75.9	haploid or aneuploid
Y 26 a/α 22		
H 1465	220.8	triploid
Y 1	269.7	triploid
Y 26	210.3	triploid

Each average value quoted is calculated from the average length and width obtained by measuring 100 random cells from a stationary phase culture. The standard deviations of the mean lengths and widths, expressed as a percentage of the mean, lay between 11% and 24%, average around 17%. This could give standard deviations of volumes within a population of cells of up to 50%.

FIGURE 15

GROWTH RATE DETERMINATIONS ON PARENT STRAINS Y 1, Y 7, Y 8, Y 9 AND Y 10



Key: ○ strain Y 1
△ strain Y 7
□ strain Y 8
▼ strain Y 9
■ strain Y 10

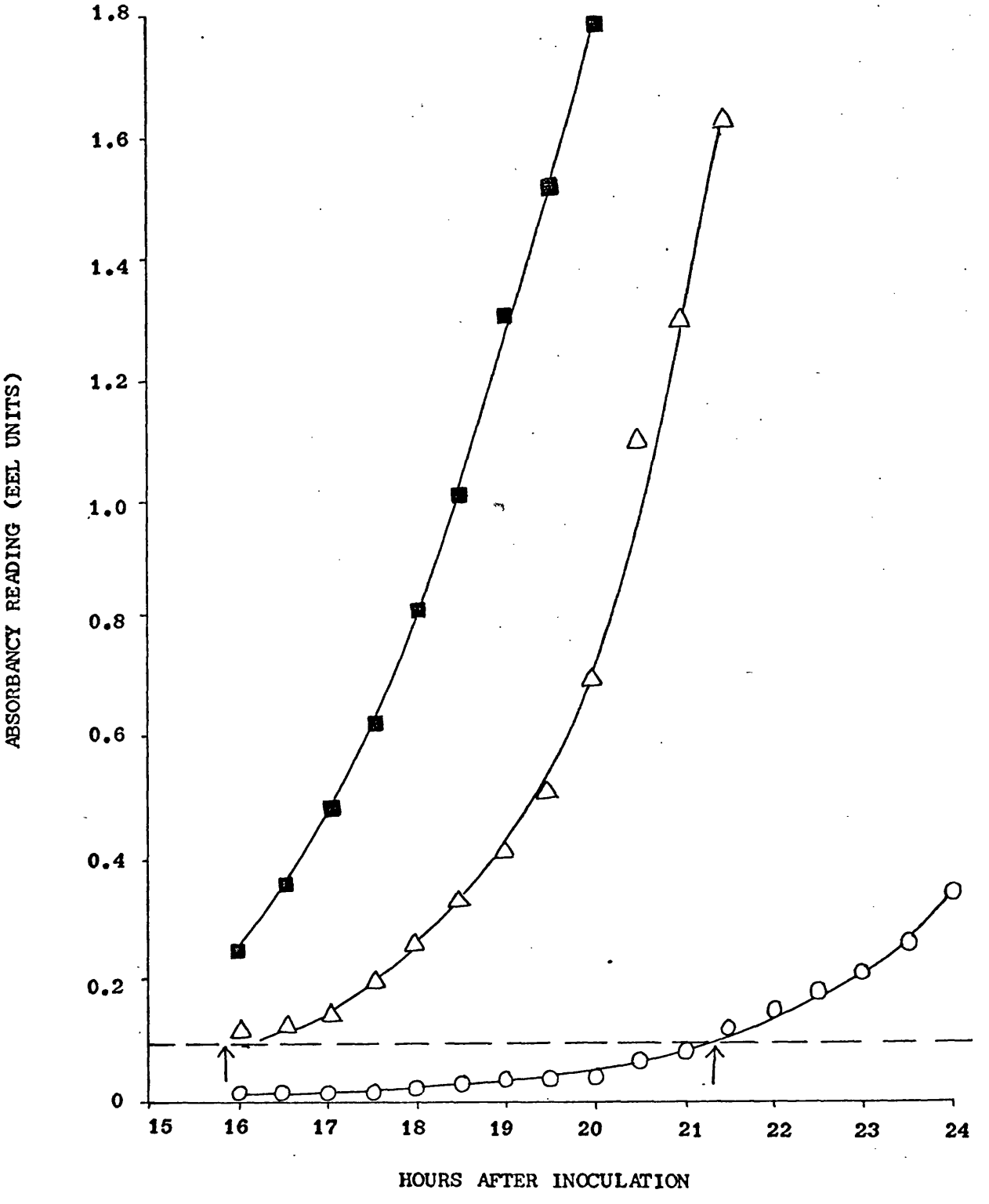
TABLE 19

Specific growth rates and mean generation times of parent and selected hybrid strains of yeast

Yeast	Experiment	Specific growth rate (h^{-1})	Mean generation time (h)
Y 1	1	0.287	2.42
Y 7	1	0.354	1.96
Y 8	1	0.375	1.85
Y 9	1	0.401	1.73
Y 10	1	0.380	1.83
Y 11	1	0.390	1.80
Y 1	3	0.415	1.67
Y 8	3	0.519	1.34
H 13	3	0.522	1.33
Y 1	4	0.517	1.34
Y 26	4	0.555	1.25
H 10	4	0.607	1.14
H 1465	4	0.512	1.36
Y 1	5, 6	0.28, 0.28	2.5, 2.5
Y 80	5, 6	0.35, 0.37	2,0, 1.9
H 1431	5, 6	0.38, 0.38	1.8, 1.8
Y 1	7	0.408	1.70
Y 26	7	0.506	1.37
H 1431	7	0.470	1.48
H 1985	7	0.396	1.75

FIGURE 16

LAG PHASE ESTIMATIONS ON HYBRIL STRAIN H 13 AND ITS PARENT STRAINS Y 1 AND Y 8



Key: ○ strain Y 1

△ strain Y 8

■ strain H 13

↑ shows the estimated end of the lag phase of growth

Growth in Wort of Hybrid Strains, Parent Yeasts and Component Mating Strains

Growth in wort of hybrids H 1431, H 1465, their parent yeasts and component mating strains, was measured by sampling periodically and estimating dry weight. The results of these experiments are given in Figures 17 and 18. The time-course of growth of the hybrid strain is like that of their flocculent parent Y 26 and the final cell concentration was far greater than that of either parent or the component mating strains.

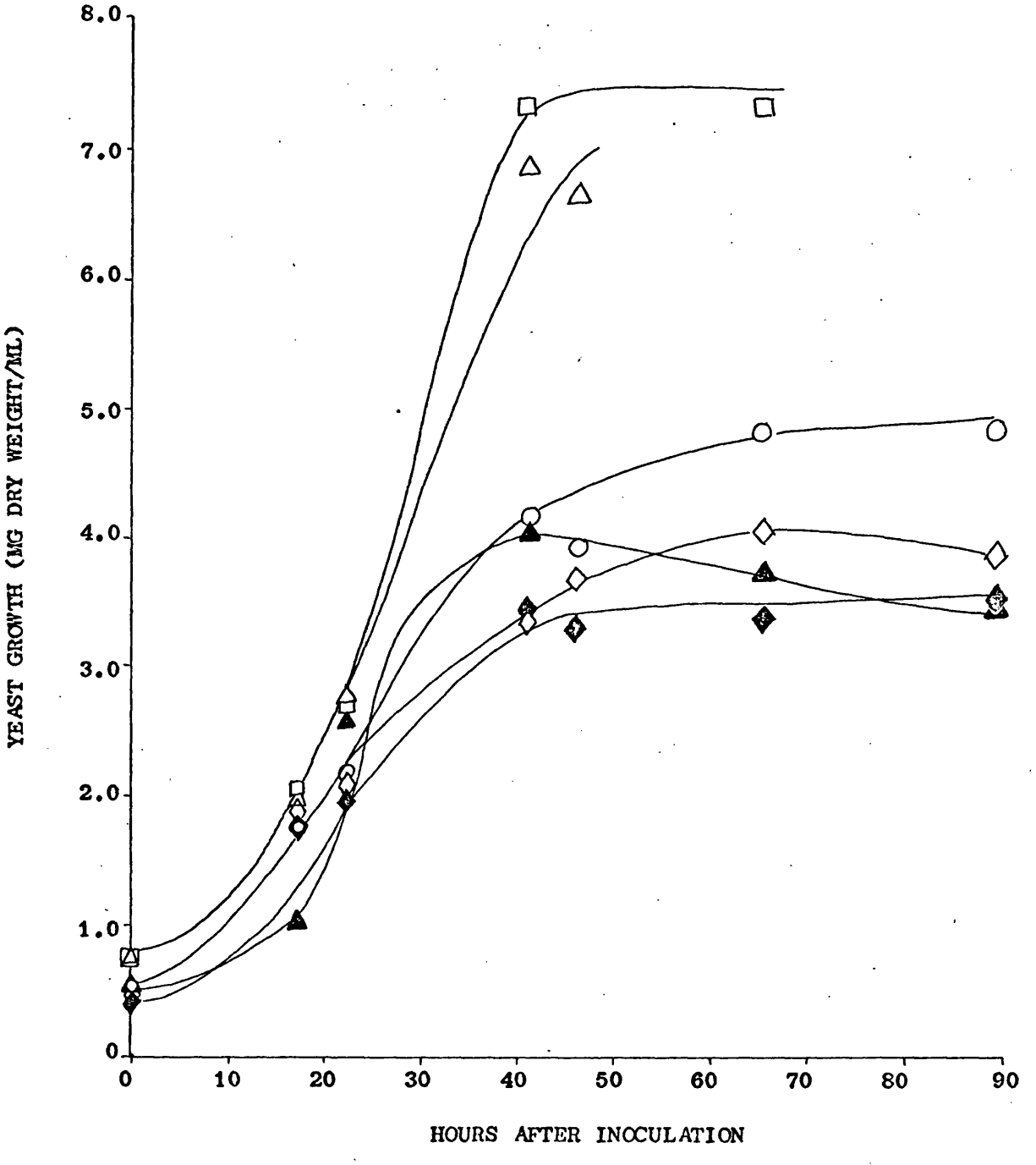
Fermentation Rates of Hybrids in Wort

When fermentation rates were first measured, consistent results could not be attained. It was thought possible that the phase of growth of the yeast strain under test, and the concentration of cells in the respirometer flasks might be critical factors, and these possibilities were investigated. It was shown that optimal fermentation rates in wort were only obtained if the cells were in the logarithmic phase of growth (Table 20) and when cell concentrations in the flask were between 1 million and 10 million cells per ml (Tables 21 and 22). Above the higher concentration the fermentation rate drops off rapidly. Therefore, in all subsequent fermentation rate determinations, logarithmically growing cultures of yeast cells were used, and concentrations per ml of one to ten million cells were included in the flasks (Methods, p 59).

The fermentation rates in wort of selected hybrids and parent yeast strains are given in Table 23. Protein contents of extracts from yeast suspensions used in fermentation rate determinations were assayed. Sample results are shown in Table 23 and presented in Figure 19.

FIGURE 17

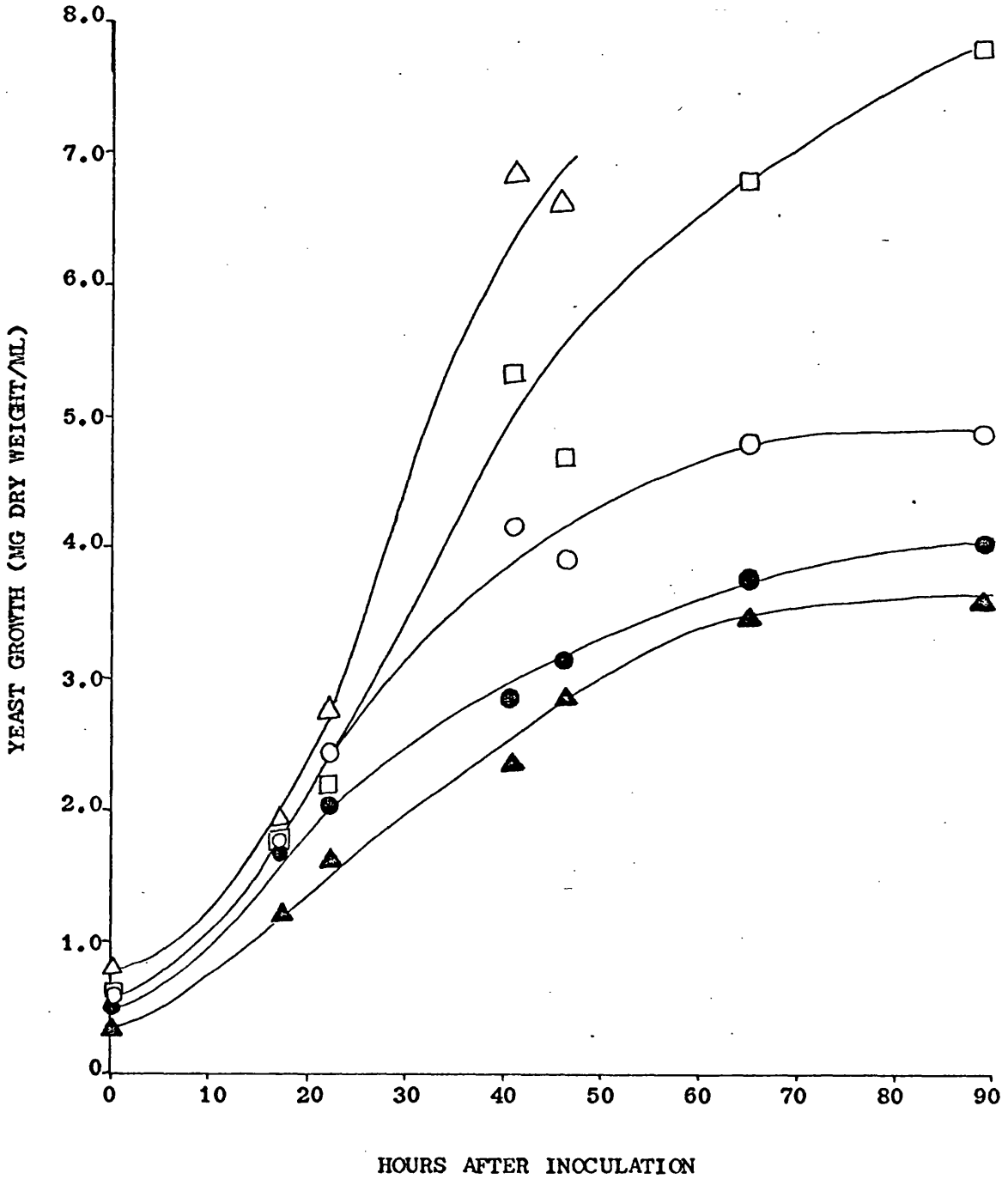
GROWTH IN WORT OF HYBRID YEAST H 1431, ITS PARENT STRAINS Y 7 AND Y 26, COMPONENT MATERS Y 7 α 2 AND Y 26 a/α 22 AND CONTROL YEAST Y 1



Key:
- ○ strain Y 1
- ◇ strain Y 7
- △ strain Y 26
- □ strain H 1431
- ◆ strain Y 7 α 2
- ▲ strain Y 26 a/α 22

FIGURE 18

GROWTH IN WORT OF HYBRID YEAST H 1465, ITS PARENT STRAINS Y 1 AND Y 26 AND COMPONENT MATING STRAINS Y 1.a.40 AND Y 26.α.23



Key:

- ○ strain Y 1
- △ strain Y 26
- □ strain H 1465
- ● strain Y 1.a.40
- ▲ strain Y 26.α.23

TABLE 20

Fermentation rates in wort, and protein contents of yeast Y 1 at different stages of growth in wort culture

Age of culture tested (h)	Phase of growth of culture tested	Fermentation rate (μl CO_2/min/mg dry wt)	Protein content (% dry wt)	Ratio: Fermentation rate / Protein content
24 h	logarithmic	7.76	42.1	0.184
24 h	logarithmic	7.95	44.6	0.178
48 h	early stationary	6.38	34.4	0.185
72 h	late stationary	2.62	30.2	0.087

TABLE 21

Fermentation rates of parent yeasts in wort at different cell concentrations

Strain number	Fermentation rates (µl CO_2/min/mg dry weight) of yeasts where the yeast count/ml of medium in the respirometer flask was;		
	10×10^6	20×10^6	30×10^6
Y 1	5.85	5.2	5.1
Y 7	7.4	6.95	6.2
Y 8	7.0	6.54	6.2
Y 9	7.0	6.4	6.4
Y 32	7.7	6.4	5.7

TABLE 22

Fermentation rates of strain Y 1 in wort at different cell concentrations

Approximate count of Y 1 (in millions/ml)	Experiment 1			Experiment 2		
	Fermentation rate (μl CO_2/min)	Dry weight (mg/ml)	Ratio: Fermentation rate / Dry weight	Fermentation rate (μl CO_2/min)	Dry weight (mg/ml)	Ratio: Fermentation rate / Dry weight
7 - 10	8.75	1.6	5.47	5.6	1.02	5.49
3.5 - 5	4.35	0.8	5.44	2.75	0.51	5.39
1.75 - 2.5	2.2	0.4	5.50	1.45	0.255	5.68
0.83 - 1.25	1.1	0.2	5.50	0.73	0.1275	5.72

TABLE 23

Fermentation rates (in µl CO_2/min/mg dry weight) in wort and protein contents of selected hybrid strains and parent yeasts

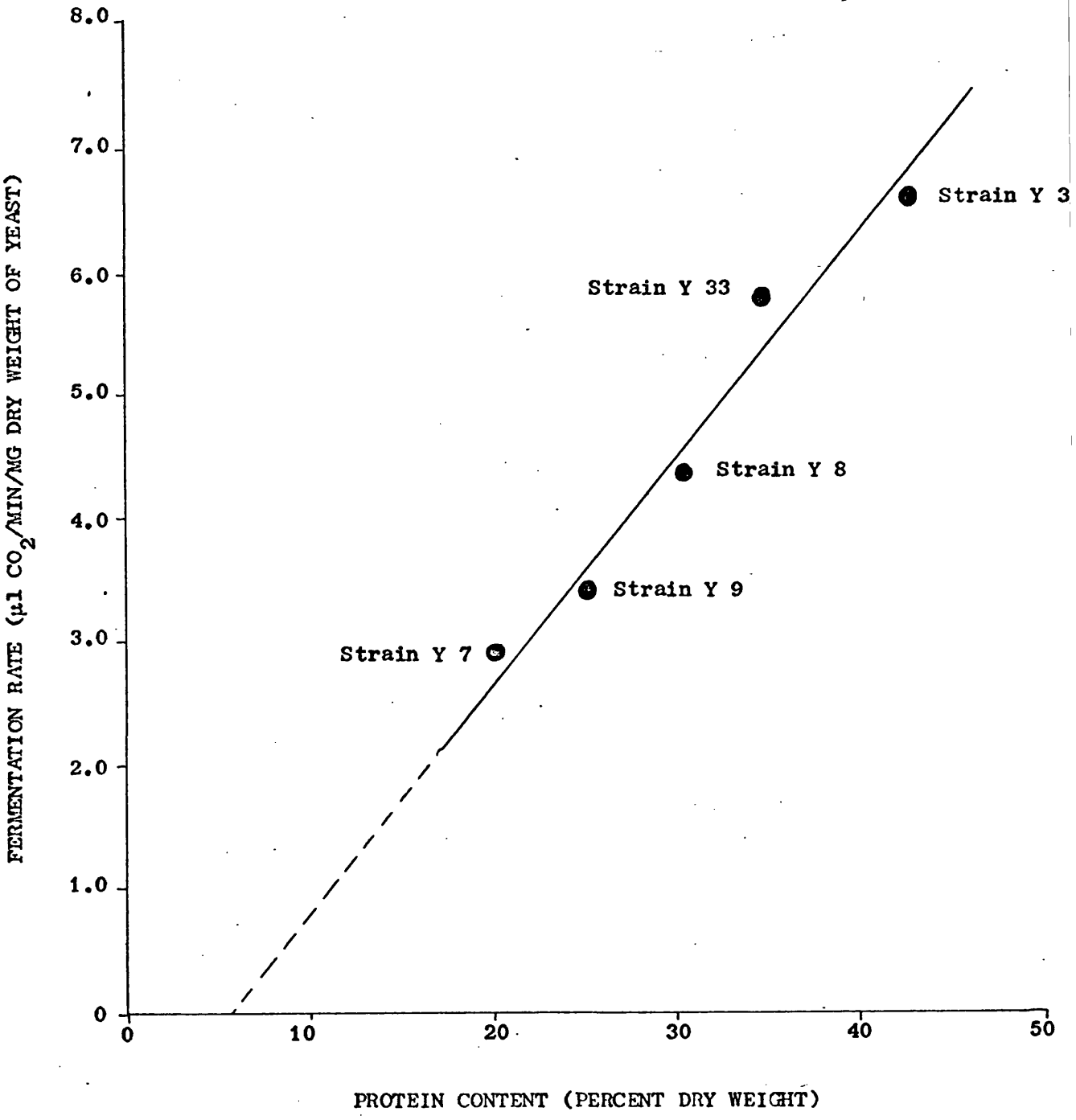
Yeast strain	Experiment number	Hybrid of yeasts (a x α)	Fermentation rates (µl CO_2/min/mg dry weight)	Protein content (percent of dry weight)
H 6	1	Y 10 x Y 8	5.8	ND
Y 10			4.6	ND
Y 8			6.8	ND
H 10		Y 10 x Y 7	6.6	ND
H 12		Y 10 x Y 7	3.6	ND
Y 7			6.1	ND
H 13	2	Y 8 x Y 1	7.5	35
Y 1			5.7	30
Y 8			6.8	32
H 14		Y 8 x Y 32	6.1	36
Y 32			8.6	50
H 13	3	Y 8 x Y 1	7.9	37
Y 1			6.6	30
Y 8			7.2	31
H 14		Y 8 x Y 32	6.4	38
Y 32			10.1	56
H 11	4	Y 7 x Y 10	4.4	ND
H 12		Y 7 x Y 10	4.0	ND
H 29		Y 7 x Y 10	4.2	ND
Y 7			7.2	ND
Y 10			8.2	ND
H 17	5	Y 8 x Y 43	6.2	ND
H 21		Y 8 x Y 43	5.4	ND
H 33		Y 8 x Y 43	3.5	ND
Y 8			5.4	ND
Y 43			5.9	ND
Y 92	6		7.9	23.4
Y 140			8.6	26.7
H 1431	7	Y 26 x Y 7	7.0	ND
Y 1			5.2	ND
Y 80			6.1	ND

ND indicates that protein determinations were not carried out on these yeast suspensions.

All protein determinations were carried out in duplicate; and the figure quoted is the average of the duplicates.

FIGURE 19

RELATIONSHIP BETWEEN FERMENTATION RATES AND PROTEIN CONTENTS OF FIVE PARENT YEASTS HARVESTED FROM WORT CULTURES IN THE LOGARITHMIC PHASE OF GROWTH



Fermentation Rates of Hybrids in Sugar Solutions

The ability of hybrid yeast H 1431, its parent yeasts and component mating strains to ferment the three main sugars in wort (glucose, maltose and maltotriose) was studied in greater detail using respirometry. The results are given in Table 24.

Characteristics of Hybrids in Production-Scale Fermentations

Large-scale fermentations using hybrid strains H 10, H 1431 and H 1465 were conducted in open vessels using strains Y 7 and Y 9 as the control mixture. The results are shown in Figures 20, 21 and 22. Hybrids H 10 and H 1465 had lag phases of similar duration, fermented at a similar rate to the control mixture and attenuated the wort better. Strain H 10 was completely non-flocculent in its behaviour. Hybrid yeast H 1431 had a longer lag phase, and fermented at a similar rate, attenuating the wort more effectively.

Hybrid strain H 1431 was also tested on a larger scale (480 brl) in a series of six trials. Only one of these trials is reported in this thesis (Figure 23). The hybrid yeast strain fermented fast and attenuated to the required level in the first two trials, but in subsequent fermentations the lag phase became extended and attenuation was poor.

TABLE 24

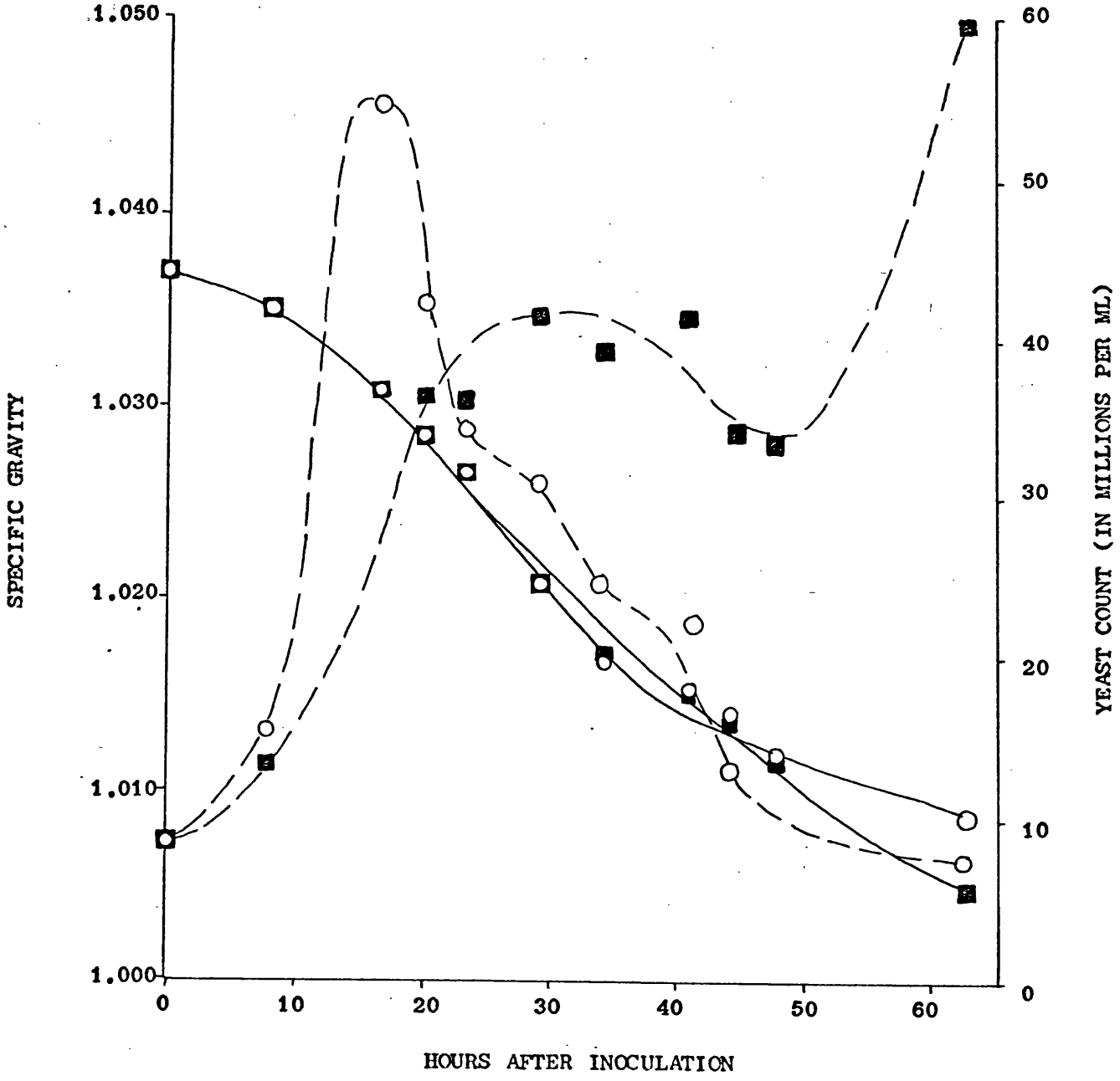
Rate of fermentation of glucose, maltose and maltotriose by hybrid H 1431, its parent yeasts and component mating strains

Yeast strain	Rate of fermentation of glucose (μl CO_2/min/mg dry weight)		Rate of fermentation of maltose (μl CO_2/min/mg dry weight)		Rate of fermentation of maltotriose (μl CO_2/min/mg dry weight)	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Y 1	1.7	3.2	2.1	2.9	0.8	0.8
Y 7	ND	3.9	1.5	2.2	0.9	1.2
Y 7 a 2	2.8	4.1	1.3	2.0	0.4	0.5
Y 26	2.7	4.3	1.1	1.5	0.7	0.8
Y 26 α 22	2.9	4.0	1.7	2.3	0.5	0.6
H 1431	2.9	4.3	2.5	3.2	0.9	1.2

ND indicates that Y 7 was not tested in this experiment

FIGURE 20

CHANGES IN SPECIFIC GRAVITY AND THE COUNT OF YEAST CELLS IN SUSPENSION DURING PRODUCTION-SCALE FERMENTATIONS OF HYBRID STRAIN H 10 AND CONTROL MIXTURE OF PARENT STRAINS Y 7 AND Y 9



○ indicates behaviour of control mixture of strains Y 7 and Y 9

■ indicates behaviour of strain H 10

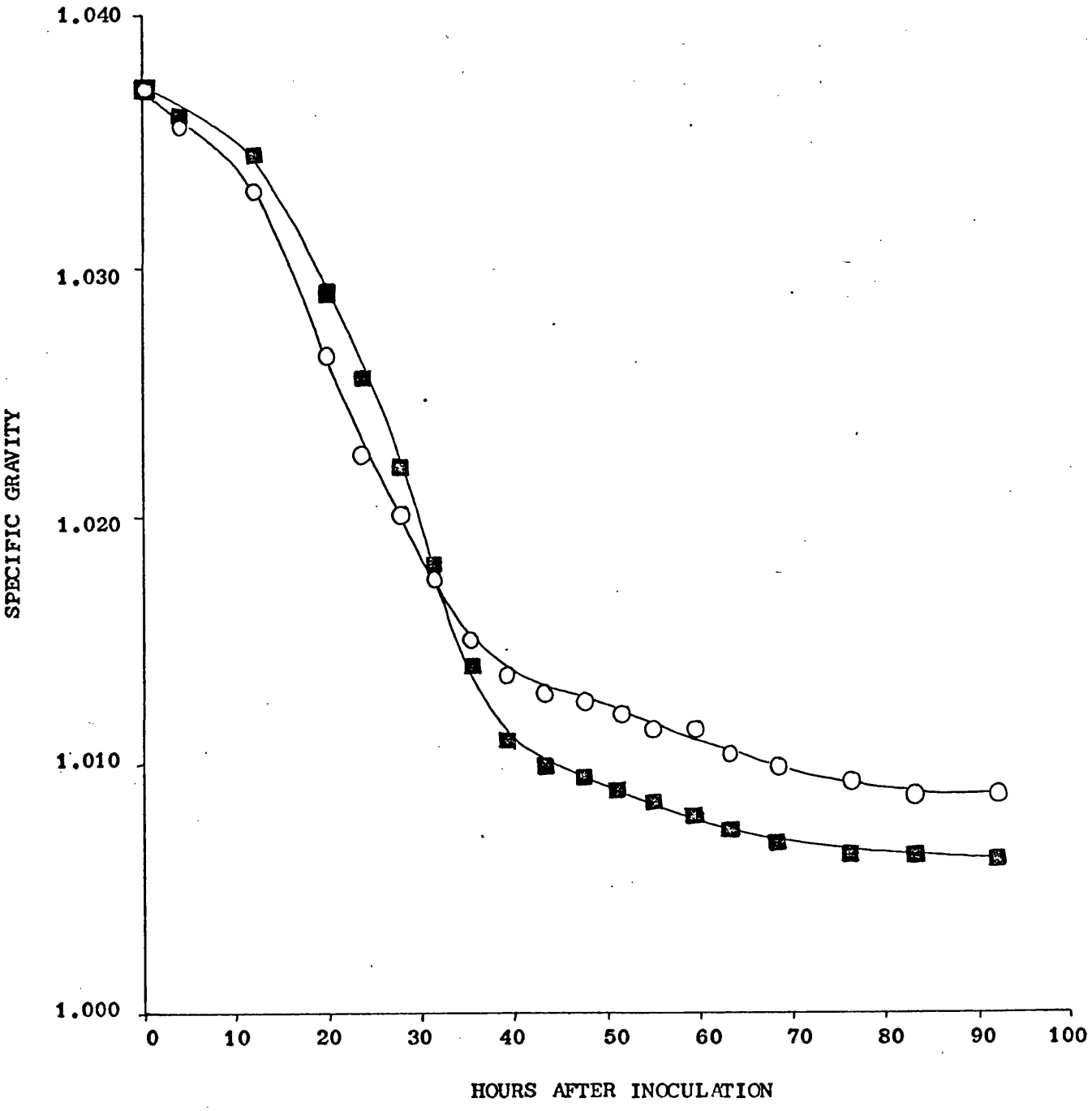
——— indicates changes in specific gravity

— — — indicates changes in the count of yeast cells

The fermentation of strain H 10 was 27 brl in capacity, and of the control mixture, 168 brl in capacity.

FIGURE 21

CHANGES IN SPECIFIC GRAVITY DURING PRODUCTION-SCALE FERMENTATIONS OF HYBRID STRAIN H 1465 AND MIXTURE OF CONTROL STRAINS Y 7 AND Y 9



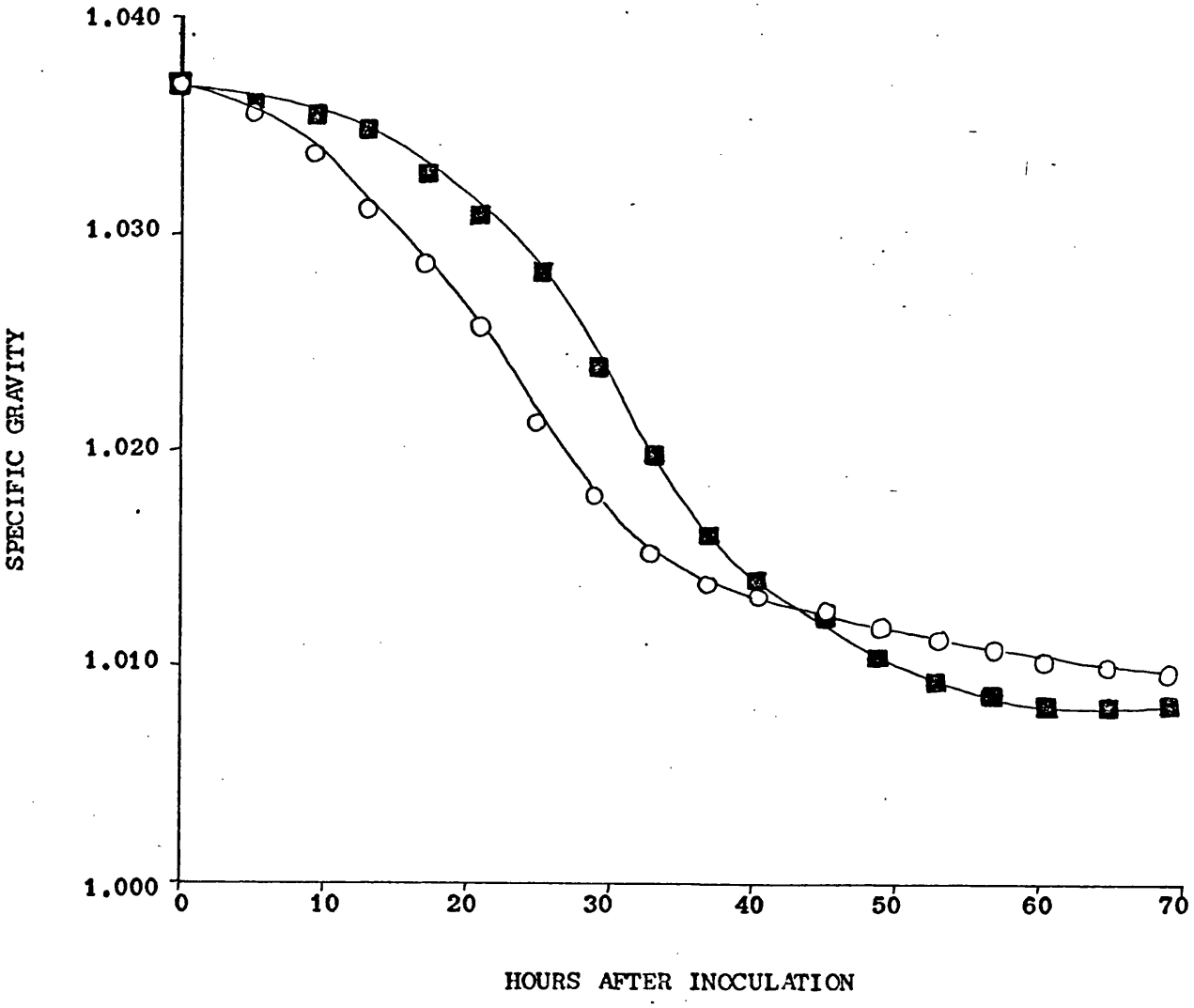
○ indicates behaviour of mixture of control strains Y 7 and Y 9

■ indicates behaviour of strain H 1465

The fermentations were 60 brl in capacity

FIGURE 22

CHANGES IN SPECIFIC GRAVITY DURING PRODUCTION-SCALE FERMENTATIONS OF HYBRID STRAIN H 1431 AND MIXTURE OF CONTROL STRAINS Y 7 AND Y 9



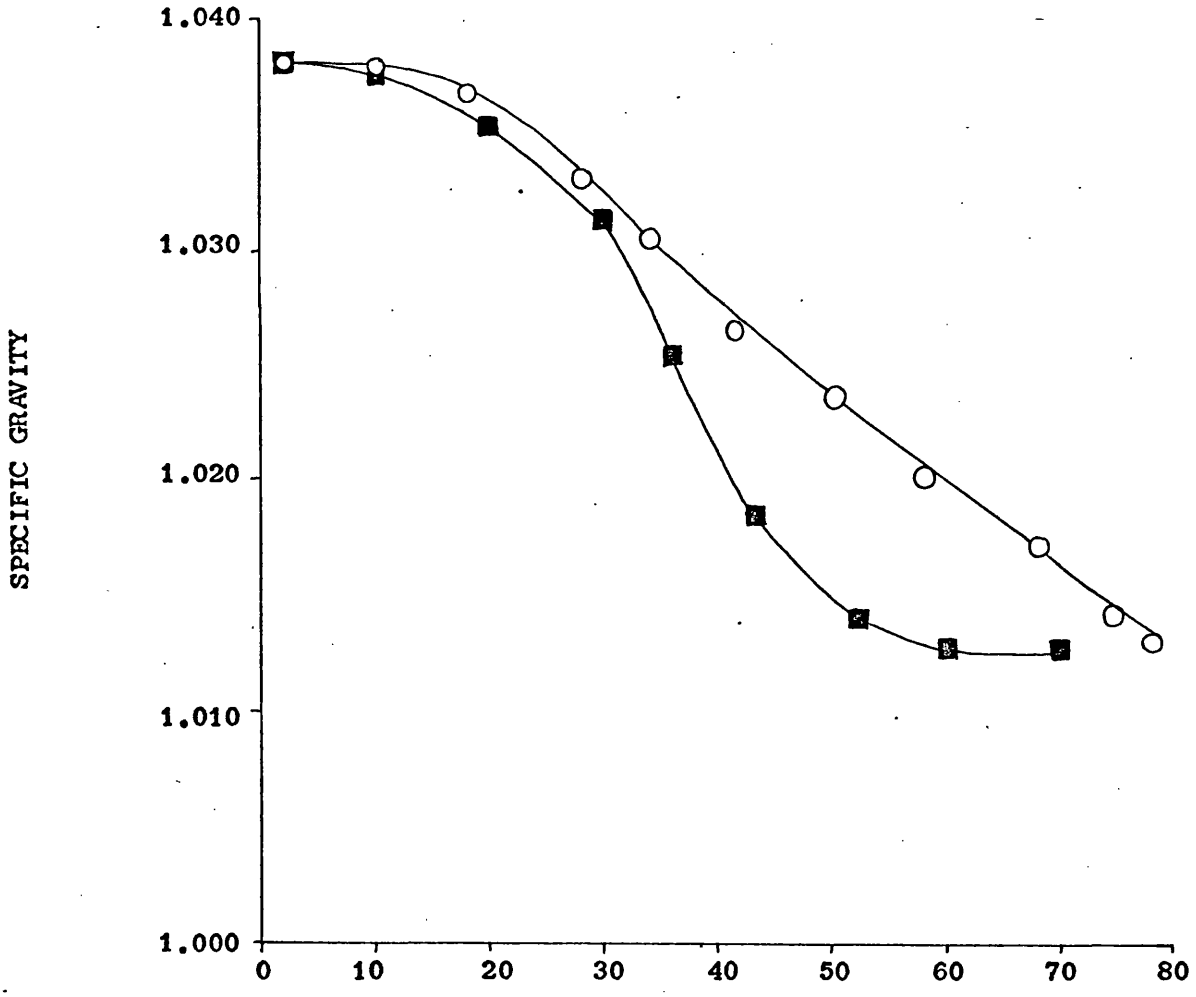
○ indicates behaviour of mixture of control strains Y 7 and Y 9

■ indicates behaviour of strain H 1431

The fermentations were 60 brl in capacity

FIGURE 23

CHANGES IN SPECIFIC GRAVITY DURING PRODUCTION-SCALE FERMENTATIONS OF HYBRID STRAIN H 1431 AND CONTROL STRAIN Y 1 (TRIAL 1)



○ indicates behaviour of strain Y 1

■ indicates behaviour of strain H 1431

The fermentations were 480 brl in capacity

The results of analyses on beers from H 1431, H 1465 and control yeasts are given in Tables 25 and 26. Analyses were not carried out on beers from the trial with strain H 10, because the beer was difficult to clarify, and contained high concentrations of yeast cells which would have interferred with analyses. Examination of results show that, in 60 brl fermentations both hybrids produced smaller amounts of total fusel oils in the beers than the control strain mixture (Table 25), and also hybrid H 1465 produced a much lower concentration of n-propanol than its control. However, considering the 480 brl fermentations of strain H 1431, there was no marked differences between the analyses of experimental and control beers from Trial 1.

Results of organoleptic evaluations of the beers from these production-scale trials are presented in Table 27. Only two trials produced beers considered to have acceptable flavours compared to the controls, the 60 brl fermentations of hybrid strain H 1431.

TABLE 25

Analyses on beers produced in 60 brl fermentations by hybrids H 1431 and H 1465 and control mixture of yeasts Y 7 and Y 9

Analysis	Trial (H 1431)	Control (Y 7 + Y 9)	Trial (H 1465)	Control (Y 7 + Y 9)
Original gravity of wort (sp. gr. units)	1.0364	1.0363	1.0370	1.0352
Present gravity of beer (sp. gr. units)	1.0064	1.0062	1.0054	1.0061
pH value	4.04	3.96	4.04	4.13
Colour (°EBC)	21.0	20.0	20.0	20.0
Alcohol (percent v/v)	3.92	3.92	4.04	3.75
(percent w/w)	3.14	3.14	3.2	2.98
Bitterness (EBUnits)	25.0	27.0	27.0	25.8
Total polyphenol content	304	300	225	203
Diacetyl content	0.12	0.15	0.06	0.15
Dimethyl sulphide content			0.18	0.23
Acetaldehyde content	17.7	4.5	1.8	7.8
3 methyl-1-butanol acetate content	1.16	0.52	1.4	0.7
Butan-2-ol content	13.6	31.4	6.8	28.4
Ethyl acetate content	23.3	10.2	24.5	32.1
3-methyl-1-butanol and 2-methyl-1-butanol content	65.0	74.6	38.9	55.3
N-propanol content	17.9	17.5	1.1	23.0
Total fusel oil content	78.5	106.1	45.7	83.7

All 'contents' are quoted as mg/litre excepting methyl sulphide which is in µg/litre.

TABLE 26

Analyses on beers produced in 480 brl fermentations by hybrid H 1431 and control yeast Y 1

Analysis	Trial 1	
	H 1431	Y 1
Original gravity of wort (sp. gr. units)	1.0382	1.0383
Present gravity of beer (sp. gr. units)	1.0129	1.0121
pH value	4.05	4.10
Colour (°EBC)	28.5	25.0
Alcohol (percent v/v)	3.3	3.4
(percent w/v)	2.6	2.7
Bitterness (EBUnits)	20.6	21.1
Diacetyl content	0.28	0.25
Dimethyl sulphide content	0.72	0.98
Acetaldehyde content	1.20	1.00
3-methyl-1-butanol acetate content	0.5	0.5
Butan-2-ol content	9.9	11.5
Ethyl acetate content	5.0	5.1
3-methyl-1-butanol and 2-methyl-1-butanol content	44.9	46.2
N-propanol content	12.8	13.1
Total fusel oil content	54.8	57.7

All contents are quoted as mg/litre excepting dimethyl sulphide which is in µg/litre

TABLE 27

Results of taste evaluation tests on beers produced by hybrid and control yeasts in production scale fermentations

Trial yeast	Control yeast or yeasts	Scale of trial (brl)	Taste evaluation method	Significance and preference	Comments on flavour of trial beer
H 10[1]	Y 7 + Y 9	60	3 glass	*SD, CP	autolysed yeast flavour
H 1465	Y 7 + Y 9	60	3 glass	*SD, NP	aged, yeasty, with an unpleasant hop character, and after-bitterness, rough, sulphury, unclean flavour
			2 glass	CP	
H 1465[2]	Y 7 + Y 9	60	3 glass	NSD	
			2 glass	NP	
			2 glass	CP	
H 1431	Y 7 + Y 9	60	2 glass	CP	Fruity or estery aroma, acceptable flavour
			2 glass	NP	
H 1431[3]	Y 7 + Y 9	60	2 glass	NP	
			2 glass	NP	
H 1431[4]	Y 1	480	2 glass	CP	unpleasant estery flavour

Measurements on fermentations producing beers numbered [1,2,3,4,5] are presented graphically in Figs. 21, 22, 23, 24 and 25 respectively

*SD indicates a one star significant difference between the beers and
NSD indicates no significant difference between the beers.
CP indicates a significant preference for the control beers and NP no significant preference for either beer.

DISCUSSION

Genetical properties of parent strains

The parent yeasts selected for this investigation include a range of strains of the genus Saccharomyces, some bakery or distillery yeasts, but in the main yeasts which are at present or which have been used in breweries. Bakery and distillery yeasts, which are strains of Saccharomyces cerevisiae, present few problems as parent strains, for they sporulate readily (Fowell, 1958) to give high proportions of four-spored asci, and spores of good viability and fertility. Brewing strains of Sacch. cerevisiae, known as 'ale' yeasts and usually capable of producing yeast 'heads' on brewery fermentations, are reported (Thorne, 1951a) to sporulate only poorly. It is worth pointing out, however, that this has not been fully substantiated by the results of a survey by Fowell (1969), for a range of values of between 0 and 70 per cent sporulation has been found among such yeast strains. The present study revealed similar values to those reported by Fowell (1969) ranging from no apparent sporulation after fourteen days on sporulation agar to 80 per cent sporulation after only three days. These parent yeasts produced two- and three-spored asci and only rarely four-spored asci. The baking and distilling strains of Sacch. cerevisiae sporulated to levels between 30 and 60 per cent, giving high proportions of four-spored asci. It is, of course, possible that higher levels of sporulation may have been attained for these yeasts, using different media. It has been reported (Fowell, 1969) that glucose, which is present in my sporulation medium, can inhibit sporulation in such strains, but preliminary unreported work in this laboratory revealed that the glucose-containing medium favoured sporulation of the more difficult strains. 'Lager' yeasts, strains of Sacch. carlsbergensis, which do not

characteristically produce yeast heads on brewery fermentations, are reported to sporulate at even lower levels, or not at all (Fowell, 1969). Emeis (1958) carried out a survey on six strains, and reported that the average level of sporulating cells was four per cent. The mean sporulation level for the brewing strains of Sacch. carlsbergensis reported in this thesis was similar to that reported by Emeis, namely 5.1 per cent for the eight strains tested. The asci, which were one- and two-spored, were produced after more than three days on sporulation medium. Yeast Y 43 has not been included in this summary, because it is a non-brewing hybrid strain, probably diploid, which produces four-spored asci like the baking and distilling strains. Of the parent strains of Sacch. cerevisiae that were examined, strains Y 9, Y 11, Y 86 and Y 311 resemble brewing strains of Sacch. carlsbergensis in that they sporulate poorly and more slowly than the other eleven strains of Sacch. cerevisiae that were tested.

A block in the ability to produce viable ascospores can theoretically occur at a number of different stages. Studies on synchronously growing cultures of Sacch. cerevisiae have indicated that the sporulation process can be divided into two major stages, namely 'readiness' and 'commitment'. During the first stage, which is brought about by a short exposure to sporulation medium, premeiotic DNA synthesis occurs, which differs from synthesis of this nucleic acid during vegetative growth in that the former utilises internal pool precursors (Simchen et al., 1972). During this period, if yeast cells are removed from the sporulation-stimulating medium, they can still revert to vegetative growth immediately. Once they have entered the 'commitment' stage, they must sporulate. Sporulation-defective mutants may exhibit no (Simchen et al., 1972) or diminished (Esposito et al., 1970) premeoitic DNA synthesis during the 'readiness' stage. It

has been demonstrated by production of binucleate cells, and the absence of cells with three or four nuclei, that blocking may occur before the second meiotic division has occurred (Esposito et al., 1970). Recent analysis of the complementation of these mutants has indicated that there are at least 50 ± 25 separate genes which can confer an asporogenous phenotype (Esposito et al., 1970). Three of the fourteen complementation groups under investigation by these workers have been shown to be dominant to the wild type. Brewing strains may therefore have mutated at one or more of these loci during their many generations of vegetative reproduction and natural selection in wort fermentations. Storage of yeasts for prolonged periods in non-metabolising conditions on growth media may also result in loss of sporulating ability (Fowell, 1969). Such mutations could account for the production of two-spored asci by the industrial strains studied in this thesis.

Premeiotic DNA synthesis is necessary for normal meiosis in yeast cells, during which the nuclei increase in size, and become, in a diploid strain of Sacch. cerevisiae, four-lobed, as spindle formation separates the four haploid sets of chromosomes. The nuclear membrane remains intact up to this stage, (Moens, 1971) and the DNA content of the cells is roughly doubled (Simchen et al., 1972). At the next stage, possibly when the cells become 'committed' to sporulation, the nuclear membrane becomes separated into four spherical sections, each containing a spore nucleus which, if derived from a normal diploid heterozygous for mating type, will contain the haploid number of chromosomes, and confer only one mating type. The double membrane around each 'prospore' (Lynn and Magee, 1970; Marquardt, 1963) may be responsible for laying down the spore wall (Beckett et al.,

1973). Failure either to pinch off the haploid genome from the four-lobed membrane of the nucleus or to produce a spore cell wall will prevent formation of mature spores, and may cause the production of three-, two- or one-spored asci. Obviously, in a brewing strain which is either triploid or aneuploid, multiplication and division of the DNA would follow a different pattern, for the meiotic divisions cannot be normal. Under these circumstances spore genomes may arise which have an incomplete complement of certain genes or even chromosomes, and are therefore incapable of producing a mature spore, either because they lack genes which determine formation of cell-wall materials, or membrane precursors, or which simply cannot metabolise the intermediates with which they are provided. Therefore asci containing less than four spores will be produced. However, proof of the production of such spores is impossible, because they do not survive beyond the ascus.

In addition to poor sporulating ability, the higher ploidy of brewers' yeast may be correlated with poor germinating ability of spores. Average spore viabilities of brewing yeasts have been quoted as 7.1 per cent for strains of Sacch. cerevisiae (Johnston, 1965) and 0.07 to 20 per cent for six production strains of Sacch. carlsbergensis (Emeis, 1958). My data, which are available only for micromanipulated cultures of selected yeasts, show values of 0 and 78 per cent viability for two strains of Sacch. cerevisiae and a range of 15 to 93 per cent viability for the three strains of Sacch. carlsbergensis. These values are higher than those quoted in the literature, but only a limited number of strains were tested. Also it must be noted that only two-spored asci of the strains of Sacch. carlsbergensis and two- and three-spored asci of the strains of Sacch.

cerevisiae were subjected to micromanipulation. The spores from one-spored asci may have poorer viabilities, but it is difficult in wet-mount preparations used for micromanipulation to distinguish them microscopically from vegetative cells.

Of the cultures obtained by germination of ascospores, with the exception of those obtained from the probably diploid derivative of Y 9 described in the Results section, between 22 and 100 per cent were incapable of mating. In the parent strains which produce no fertile maters or which cannot sporulate at all, presumably the genetic material segregates so abnormally at meiosis that the mating type alleles never segregate singly. Yeasts Y 80 and Y 307, strains of Sacch. cerevisiae, produced a significantly high proportion of sterile strains, a characteristic of lager yeasts (Emeis, 1958). It is interesting to note that these, and also the non-sporulating 'ale' yeasts Y 86 and Y 311, flocculate to the base of the vessel during a brewers' wort fermentation, like strains of Sacch. carlsbergensis, and do not form flocs which rise to the top of the fermenting liquid, which is the normal behaviour of 'ale' yeasts. These strains which therefore behave like bottom-fermenting 'lager' yeasts, are more flocculent than most production strains of Sacch. carlsbergensis. They can be considered to be intermediate types.

In addition, twelve of the parent yeasts produced a proportion of mating strains capable of combining with either of the a or α standard types used for mating type determinations. This phenomenon has been reported previously (Lindegren and Lindegren, 1943b; Lindegren, 1945). As yet, no explanation has been forthcoming as to the origin of these a/α strains, but it is probable that they are 'aneuploid' (see cell size data,

Table 6) with an additional chromosome or chromosome fragment carrying the gene determining the second mating type. This might explain why these strains, when mated, often have a preference for strains of one mating type, only mating with strongly fertile strains of the other type. In fact, of the 26 a/α mating strains which were compatible with maters from the industrial yeasts, seven in practice behaved as pure a or α strains, and four had a strong preference for one mating type.

Further examination of the proportions of a and α mating types reveals that most of the parent strains of Sacch. cerevisiae are heterozygous for mating type, producing both a and α mating strains. The derivative of strain Y 9 designated Y 9-1, however, produced mating cultures only of the a type, and is therefore either homozygous for mating type, or produced α mating strains which were non-viable for some reason. However, it sporulates well, which is not usual for yeasts which are homozygous for mating type. It is also likely that some of the yeast cells derived from spores of this strain were capable of self-diploidisation (Roman and Sands, 1953), that is, carried the D-gene (Winge and Roberts, 1949) and became diploid, for some of the spore isolates obtained could both sporulate and mate. Such yeasts are termed 'illegitimate diploids' (Lindegren and Lindegren, 1943c).

Among the strains of Sacch. carlsbergensis, Y 43, the non-brewing strain, and Y 93 are both heterozygous for mating type. The other two 'lager' yeasts, Y 41 and Y 140, from which 'mater' cultures have been derived using micromanipulation, can probably be considered as homozygous for mating type like Y 9, and again of the aa or aaa type. Although these strains did produce mating strains which could mate with the a standard

strain, a highly fertile yeast, they would not combine with a strains derived from other parent yeasts. This agrees with reported results for brewing strains of Sacch. carlsbergensis (Emeis, 1958). However, it is difficult to understand how Emeis concludes that his tested strains of lager yeasts were homothallic, that is, homozygous for mating type, for he obtained no fertile mating strains of either mating type from them, because their sporulating ability was too poor. This conclusion was apparently based only on their poor sporulating ability. In addition some of his data for mating yeasts derived from strains of Sacch. cerevisiae did not fit well with expected ratios for triploid yeasts, although they were all above the acceptable five per cent level. Some of the ratios he obtained might have been better explained by the hypothesis that a number of the brewing yeasts he tested were aneuploid.

The homothallism of these yeasts (Y 41 and Y 140) would account for the fact that they are capable of mating with 'α' mating strains. Such mating ability of yeasts with a ploidy above n has been noted before (Hartwell, 1970). A similar procedure has been commonly used to produce artificial polyploid strains of yeast for genetic studies (Mortimer and Hawthorne, 1966). However, homothallism has been said to prevent a yeast from sporulating (Roman et al., 1955; Leupold, 1956 a and b) because it may cause abnormal segregation of chromosomes. This is not strictly true, as has been shown for a strain of Sacch. chevalieri (Winge and Roberts, 1949), for chance meiotic events may give rise to viable spores. Emeis (1958) achieved sporulation of bottom-fermenting strains of brewers' yeast which he assumed were homozygous for mating type. In my study, up to five per cent of the brewing strains of Sacch. carlsbergensis, which are commonly

homozygous, sporulate after a prolonged period on a sporulation medium. This emphasises the importance of the type of medium used for sporulation studies, for most strains can be induced to sporulate to a limited extent if their environment is appropriate.

It is possible to calculate from spore segregation patterns the likely ploidy of polyploid yeasts. Genetic analysis of mating products has indicated that some strains of brewing yeasts are triploid (Emeis and Windisch, 1960; Johnston, 1963) and others aneuploid (Windisch, 1961), or possibly allopolyploids (Fink, 1971). All these conditions confer low sporulating ability on the strains, and poor fertility and viability on their spore derivatives (Windisch, 1961). Because of their uneven chromosome numbers, abnormal segregation of chromosomes occurs during the meiotic divisions, producing asci containing only one, two or three spores, formation of the other spores being incomplete because they have not their full genetic complement.

However, it is difficult to assess the ploidy of all the brewery yeasts investigated in this study from the proportions of a, α and sterile mating cultures obtained from them, because in some cases the numbers of spore isolates were small. The percentages of sterile mating strains derived from strains Y 1, Y 26, Y 80, Y 304 and Y 307 indicate that these yeasts at least are not simply diploid, but either triploid or aneuploid of $(2n + x)$ or $(3n + y)$ constitution where n is the haploid number, and x and y are unknown numbers of extra chromosomes or chromosome fragments less than n. However, other 'ale' yeasts, namely Y 7, Y 8 and Y 10, produce ratios of mating strains (a:α:sterile) closer to the 3:1:2 or 1:3:2 ratios expected for triploid yeasts. Additional evidence for the

uneven ploidy of these strains is demonstrated by cell size estimations (Tables 6 and 18), for yeast strains of ploidy higher than 2n characteristically give rise to spores which are haploid, aneuploid or diploid, and therefore spore cultures of widely differing average cell sizes. In addition sporulating ability of parent strains and the viability of the spores produced can indicate whether a strain is polyploid or diploid, since both become poorer as the ploidy rises.

Baking and distillery yeasts are said to be either diploid (Fowell, 1958) or triploid (Emeis, 1958). Those strains tested in this study produced a proportion of sterile maters, which is not to be expected from normal diploid strains. In addition, the proportions of a to α to sterile mating strains for yeast Y 32 closely approximates to the 3:1:2 ratio for an aaα triploid yeast.and, theoretically, diploid yeasts should not produce a/α mating strains. The a/α strains were ignored when calculating the ratio.

The bipolar mating system of Saccharomyces yeasts, which are members of the Ascomycetaceae, indicates that a mating strains will always hybridise with α strains. This is not true, for a further factor, namely compatibility, determines whether an expected cross will actually occur. Compatibility can be expressed as the number of actual matings that take place between the appropriate mating strains derived from two parent yeasts divided by the calculated number of possible hybrids that could be formed. However compatibility is also biased by the change of mating strains into sterile forms (Lindegren and Lindegren, 1944). Examination of the compatibilities of brewing and non-brewing strains of Saccharomyces

with each other reveals that the system in general favours the 'out-breeding' of yeasts, strains of 'ale' yeasts being more compatible with 'lager' strains than with their own kind, and both being even more compatible with the non-brewing strains (Table 12).

As already mentioned (p 133) α strains from Y 41 and Y 140 mated only poorly with the standard strains and would not mate at all with the a cultures derived from parent ale strains. The third strain of Sacch. carlsbergensis (Y 93), which was subjected to micromanipulation, produced mating strains of which only the a types mated effectively. However the mass spore-isolation technique gave rise to a single a/α mating strain from this parent yeast which mated as an α type in all but one cross, which was one with an exceptionally fertile α strain from Y 33.

Physiological properties of hybrid strains compared with parent yeasts

Obviously the genetic basis of each of the fermentation characteristics tested in the small-scale fermentations is complex, and must be controlled by more than one gene pair. Flocculence is probably governed by at least three polymeric gene pairs (Thorne, 1951b). This worker selected four-spored asci from sporulating cultures of hybrid strains, and examined the flocculence characteristics of their ascospores. The observed numbers of these asci which segregated each possible ratio of flocculent to non-flocculent spores, did not, in all cases, agree well with the theoretical values obtained by calculation. Thorne (1951b) explained the divergencies from the expected values by proposing that the mutation rate of genes conferring flocculence to those determining non-flocculence may occur at a very high rate. However, it is possible that

his assumption of a three-gene system was incorrect for these yeasts. His observed numbers might be better explained, if one considers that some strains may contain four or more polymeric genes governing flocculence characteristics.

It is possible that gene dosage may account for the different degrees and types of flocculence found among brewing yeasts. This would explain how hybrids of greater flocculence than either parent strain were produced in my study. The gene-dosage effect was discounted by Thorne (1952) but it must be emphasised that the test that he used to differentiate between flocculence characteristics of yeast strains is based on sedimentation, not flocculence, and probably does not differentiate well between yeasts of flocculence types III and IV. Such gene-dosage effects have also been found among other genetically controlled systems in yeasts, namely the maltase- and invertase-synthesising systems. There is some evidence for this dosage effect where mating strains and derived hybrids have been examined for flocculence behaviour, and two poorly flocculent mating yeasts combine to form a more flocculent hybrid. However, it must be emphasised that it can be difficult to estimate the flocculence characteristics of mating strains. This is because growth of haploid cultures can give false results (Townsend and Lindegren, 1954), for they form large 'chains' or clumps of cells which can behave in a similar manner to flocs. Separation of cells is therefore necessary before flocculence determinations can be made.

Although flocculence is reported to be a dominant trait (Gilliland, 1951; Thorne, 1951b, 1952), overall, only a small proportion of hybrids (two

per cent) produced in my study were more flocculent than either parent, and most of these were derived from crosses between strains Y 1 and Y 304 (49 per cent of these hybrids), Y 8 and Y 10 (seven per cent) and Y 8 crossed with Y 43 (four per cent). Most of the remaining hybrids had flocculence characteristics similar to, or intermediate between, those of their parent strains. However, a large number of crosses gave rise to a proportion of hybrid strains (13 per cent of the total number), which were less flocculent than either parent. These were those between mating strains derived from Y 1 crossed with those from Y 80 and Y 304 (10 per cent and 31 per cent respectively), crosses between Y 7 and Y 26 maters (four per cent), Y 26 and Y 80 maters (eight per cent), Y 304 mating strains with Y 26 and Y 80 maters (32 per cent and 45 per cent respectively) and Y 26 and Y 41 maters (10 per cent). Strain Y 9 produced mating strains which gave non-flocculent hybrids, and these values have not been included in the calculation of the 13 per cent. It is therefore evident that poorer flocculence is a more common event than improved flocculence, possibly because less-flocculent strains have faster growth rates and hence would be selected. These values are obviously too high to be explained by simple mutation in flocculence genes to give genes conferring non-flocculence. An even larger proportion (235 out of a total number of 252) of second generation hybrids, produced from mating strains derived from H 94, H 95, H 96, H 98 and H 99 with those from 'ale', 'lager' and bakery yeasts, were non-flocculent (Table 15).

The anomalous behaviour of strain Y 9, which although of type III flocculence itself, produced non-flocculent mating strains that conferred poor flocculence on their hybrids might be explained in a number of ways.

Firstly those spore isolates which can mate may carry a gene determining production of a repressor molecule acting on the genes determining flocculence. It is also possible that the flocculence genes conferring Group III flocculence on the parent strain may be linked to the $\underline{\alpha}$ mating type, and therefore lost in all of the mating cultures and hence their hybrids. It is worth noting that the $\underline{\alpha}$ gene has been mapped, and is on chromosome III with one of the genes determining the fermentation of maltose (Mortimer and Hawthorne, 1966, 1973).

Turning to the head-forming ability of hybrid strains, it can be seen that most of them have the ability to form yeast heads as readily as their parents or their ability to do so is intermediate, with only six per cent producing smaller heads, and 3.6 per cent larger ones. This characteristic must, obviously, be governed by a number of genes, since hybrids can be obtained which have head-forming characteristics very different from their parent strains (for example, non-head forming hybrids from head-forming parent yeasts Y 1 and Y 7, and strong-head forming hybrids from bottom-fermenting strains Y 26 and Y 80). It is again possible that factors which control the formation of a yeast head are additive in effect. Examining the head-forming abilities of hybrids and comparing them with their flocculence behaviour, it is evident that the capacity to form a head is not linked with any particular type of flocculence characteristic. A more detailed microscopic examination of the chain- and floc-forming behaviour of the yeasts, as in the technique of Gilliland (1957), might throw more light on this subject.

A large proportion of the hybrid strains produced in my study (19 per cent, Table 14) have poorer attenuative ability than either parent strain,

and only a few (five per cent) were able to ferment brewers' wort to a lower specific gravity than their parents. It is widely believed that attenuative ability of a yeast strain is related to the efficiency of the system governing the fermentation of maltose and maltotriose. This would indicate that a high proportion of the hybrids may have a less efficient maltotriose permease system, or that their polymeric gene system controlling fermentation of maltose and maltotriose (Oeser and Windisch, 1964) has fewer genes, that is, lower gene dosage, than their parent strains. An alternative explanation is that the physical behaviour of yeast cells during fermentation may influence their ability to ferment wort down to a low attenuation gravity. When the attenuative ability and flocculence characteristics of hybrids were examined (see Appendix I), it was obvious that, where a high proportion of hybrids are flocculent (that is, in Groups III and IV), there is a larger percentage of hybrid strains which have medium or poor attenuative ability. Where large numbers of hybrids produced from one pair of parent strains are poorly- or non-flocculent (Groups II and I respectively), a similar proportion of those yeasts have good attenuating properties. This finding is to be expected for, when yeast cells flocculate, they are removed from suspension during fermentation and are less able to ferment the remaining saccharides in the wort. It is not true, however, to say that all poorly flocculent yeasts are attenuative, or vice versa for, as already mentioned, attenuative ability is controlled by other factors, including the polymeric gene systems responsible for the fermentation of maltose and maltotriose.

The duration of the lag phase of growth of the majority of hybrid strains is similar to, or intermediate between, those of their parent

yeasts, but a proportion (18 per cent) had a longer lag and a few (1.8 per cent) a shorter lag phase than either parent strain. The duration of lag phase in a brewery fermentation is controlled by a number of factors. The time taken for yeast flocs to become dispersed and the period of time taken for essential genes determining the production of inducible enzymes to be derepressed are the most important of these. However there is no evidence that any of the enzymes that catalyse reactions involved in sugar fermentation are inducible, although some of the genes that govern synthesis of enzymes involved in growth almost certainly require de-repression. In addition, yeast cells, grown under near anaerobic conditions, such as a brewery fermentation, cannot always synthesise sufficient membrane constituents for active growth to occur. For example, an important membrane component, namely ergosterol, required for growth under anaerobic or microaerophilic conditions, can only be synthesised in the presence of oxygen (Andreasen and Stier, 1953). Synthesis of such compounds must occur early in a brewery fermentation, when oxygen is plentiful, so that growth can proceed when supplies of the gas become depleted, and this period of synthesis will form part of the lag phase of growth.

Cells of a strongly flocculent type, when re-inoculated into fresh brewers' wort, take an extended time to disperse. Such a yeast strain often has a prolonged lag phase of growth because, until dispersed it cannot ferment optimally. Examining the results summarised in Appendix I, it is apparent that, where a large proportion of hybrids are of Group III or IV flocculence, a small proportion only of the total hybrids have short lag phases. Also where a majority have been designated as having poor flocculence (Group I and II) a similar proportion have short- or medium-

length lag phases. However, as with attenuative behaviour, there are some notable exceptions. For example, among the hybrids between yeasts Y 1 and Y 9, all non-flocculent, there are a proportion of hybrids (16.7 per cent) with long lag phases, like strain Y 1. All hybrids derived from parents Y 1 and Y 26 were flocculent, but 10.8 per cent have short lag phases. Very few crosses seem to give a proportion of hybrids with lag phases shorter than either parent yeast strain - only those between mating strains derived from Y 1 and Y 304, Y 1 and Y 93, and Y 1 and Y 140 - all of which are crosses between top-fermenting flocculent yeast Y 1 and bottom-fermenting strains which are poorly flocculent.

It is apparently a more common event for strains with longer lag phases, that is with extended periods for de-repression of enzyme synthesis, to be produced. Eighteen out of the 59 possible different combinations of mating strains derived from two parent strains gave a proportion of hybrids with this less favourable trait. In fact, overall trends among hybrids are towards less desirable behaviour, for overall more hybrids have also been prepared which have poorer attenuative ability than their parent strains.

Similar divergencies of behaviour of hybrids were demonstrated when the fermentation capacity of selected strains was examined respirometrically, for some ferment wort at a faster rate than either parent, and others more slowly (Table 23). This again emphasises the additive and subtractive effect of the polymeric genes controlling the fermentation of the di- and tri-saccharides in wort. Gene dosage can explain how hybrid strains with faster fermentation rates than either parent yeast can be produced. The parent strains can give rise to mating strains each con-

taining genes for the production of maltase (α-D-glucoside glucohydrolase), presumably not all at the same loci in the two compatible mating strains, and therefore the hybrid can contain more genes for maltase production than either parent yeast. Production of hybrid offspring with poorer fermentation rates than either parent indicates that at least one of the gene loci determining fermentation of maltose and maltotriose are present in the heterozygous state in one or both parent strains. This would mean that mating strains could be produced with one or more of these fermentation genes absent.

There is also an interesting link between fermentation rate and protein content, which seems to be proportional for yeast cells grown under the same conditions, in the logarithmic phase of growth (Figure 19). This agrees with Thorne's findings of a relationship between fermentation rate and yeast nitrogen content (Thorne, 1954). There is also a correlation between the protein content of a single strain of yeast and its fermentative capacity at different stages of growth. Examination of the figures quoted in Table 20 shows that one can therefore extrapolate back to a basal protein level at which fermentative capacity of a yeast strain is nil. This could presumably represent a level of structural proteins and constitutive enzymes in the yeast cell. The ratio of fermentation rate to protein content is not constant for a particular batch of medium unless all the yeasts are in the logarithmic growth phase, and is therefore unlike the 'fermentation efficiency' of Thorne (1954).

Selected hybrids were examined in greater detail for their fermentation rates in defined media. Examination of the abilities of hybrid strain H 1431, its parent yeasts and component mating strains, to ferment

the main saccharides that occur in brewers' malt wort showed obvious differences. The hybrid yeast fermented glucose as rapidly as the more flocculent parent Y 26, maltose more readily than either parent, and maltotriose as well as the less flocculent parent, under shaken anaerobic conditions in the respirometer. Similar improvements have already been explained in this Discussion in terms of the gene-dosage effect. The hybrid H 1431 also had a similar maximum specific growth rate in wort to its parent strain Y 26, a characteristic which may be linked to the rate of fermentation of glucose (Table 19). These data would indicate that strain H 1431 should attenuate wort well, and ferment fast, which was shown in laboratory-scale tests and production trials (see Figures 8, 13, 22 and 23, and Tables 16 and 17). The efficiency of the strain is accentuated by the fact that it, and also hybrid H 1465, both reach higher cell concentrations earlier in wort fermentations than either of their parent strains (Figures 17 and 18). Unfortunately the full attenuative potential of strain H 1431 cannot be realised because of its excessive flocculence, which would be increased by the brewery practice of 'bottom-cropping', that is, harvesting the yeast from the base of the vessel for repitching.

It is also evident that composition of a pregrowth medium can have a marked effect on the rate of fermentation of sugars, as shown in the difference between the values for the two experiments in Table 24. These experiments were carried out under exactly the same conditions except that different batches of ale wort were used to grow up the yeast strain before the experiment. Such differences in behaviour of yeasts are commonly noticed in breweries, where changes in malt quality may decrease or increase fermentation time to significant extents.

The results obtained in these experiments can also be used to calculate the length of each phase of fermentation in wort, assuming average counts of ca 25 million cells per ml during the period of fermentation of glucose and maltotriose, and approximately 50 million cells per ml while maltose is being metabolised. Approximate concentrations of the sugars in brewers' ale wort of sp. gr. 1.040 are one per cent glucose, four to five per cent maltose and one per cent maltotriose (MacWilliam, 1968). In addition it must be noted that the rates of fermentation of glucose are not necessarily a true representation of the rates in wort, because, for the purposes of my experiments, a concentration of four per cent glucose was used. Calculations from the results for control strain Y 1 in the second experiment, however, give a period of glucose fermentation of around 10 hours, of maltose fermentation, 26 hours, and an estimated duration of maltotriose metabolism of approximately 42 hours, giving a total fermentation time (assuming the sugars are used sequentially), of approximately 78 hours. This value agrees well with the reported actual brewery fermentation time of between 70 and 80 hours (Fig. 23). For hybrid strain H 1431, these values can be estimated at 8, 24 and 28 hours respectively, totalling 60 hours, a figure close to that attainable in preliminary production-scale trials using this hybrid (Fig. 23). It must be emphasised that in these respirometric experiments the effect of flocculence would have been eliminated by shaking.

As mentioned before, a most important consideration when selecting a yeast strain for use in a brewery is the flavour that the strain produces in a beer. Analyses of the beers produced by experimental yeasts reveals

that, compared with the control mixture Y 7 and Y 9, which is used in production at one brewery in this country, the hybrids gave rise to decreased concentrations of total fusel oils (the sum of the concentrations of butan-2-ol, 3 methyl-1-butanol and 2 methyl-1-butanol) and higher concentrations of 3-methyl-1-butyl acetate. Production of fusel alcohols is linked to nitrogen metabolism, for higher concentrations are produced when nitrogenous nutrients in the wort become limiting (Äyräpää, 1973). It is possible that the two hybrid strains, H 1431 and H 1465, have a somewhat different nitrogen metabolism or faster amino-acid turnover rates than the control yeast strains, and hence remove nitrogen-containing compounds from the wort more slowly. Ester production is linked with lipid metabolism, for unsaturated 9(cis)-fatty acids strongly depress the formation of these compounds (Äyräpää, 1973), and their saturated counterparts stimulate the production of acetate esters. Therefore the hybrid yeasts may also have altered ester metabolism, when compared with the control strains. It is possible that the fusel oils may become esterified into acetates, but the mechanisms are not yet clear. It is likely that changes in the constitution of membrane systems within yeast cells alter the metabolism concerned with ester formation.

There is no evidence in the analytical figures for the 'unpleasant estery' flavours quoted for beers produced by strain H 1431 in the largest scale fermentations so far conducted. It must be realised that until every compound present in beer can be identified and quantified it will be impossible to relate the production of these minor components with organoleptic evaluations. It is, after all, the ultimate purpose of a selected brewing yeast to produce a palatable beer of consistent quality.

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APPENDIX I

APPENDIX 1

Distribution of fermentation characteristics among hybrid strains and their parent yeasts

Flocculence type, (Flocc)

I	non-flocculent	
II	fairly flocculent	- AB 304 type flocculence
III	flocculent	- AB 1 type flocculence
IV	very flocculent	- AB 80 type flocculence

Head formation, (Head)

-	no head
±, +	foam or slight head
++, +++	medium or good head

Attenuation, (Att)

Good (G)	final sp. gr. > 0.0015 lower than that attained by Y 1
Medium (M, Med)	final sp. gr. = that attained by Y 1 ± 1.0015
Poor (P)	final sp. gr. > 0.0015 higher than that attained by Y 1

NB. The highest final sp. gr. obtained normally is ca 1.020, and the largest standard deviation expressed as a percentage of the mean is 4%. Therefore 0.0015 sp. gr. units lie outside the errors of the experiment, and indicate a difference in behaviour.

Lag phase, (Lag)

Short (S)	24 h. EEL reading > 0.7 units more than that attained by the Y 1 culture
Medium (M, Med)	24 h. EEL reading = that attained by the Y 1 culture ± 0.7 units
Long (L)	24 h. EEL reading > 0.7 units less than that attained by the Y 1 culture

NB. The highest 24 h. EEL reading obtained is ca 5.0 units, and the largest standard deviation expressed as a percentage of the mean is 11%. Therefore 0.7 units lie outside the errors of the experiment and indicate a difference in behaviour.

Number of hybrids:-

No. indicates the total number of hybrids produced between the mater strains of the two yeasts.

76 T, 11 T, etc. indicate the number out of the total hybrids between two yeasts actually tested.

APPENDIX 1. CONTINUED

APPENDIX 1

1. Hybrids of brewery strains of Saccharomyces cerevisiae

Hybrids of yeast strains	Number of hybrids	Percentage of hybrids with:- Flocculence type:-				Head formation:-		
		IV	III	II	I	-	±,+.	++, +++, etc.
Y 1 x Y 7	3	0	66.7	33.3	0	33.3	0	66.7
Y 1 x Y 8	2	0	0	100	0	50.0	0	50.0
Y 1 x Y 9	6	0	0	0	100	33.3	0	66.7
Y 1 x Y 10	1	0	100	0	0	0	100	0
Y 1 x Y 26	69 68 T	86.1	13.9	0	0	61.5	17.0	21.5
Y 1 x Y 80	66 65 T	64.6	24.6	10.8	0	80.0	6.2	13.8
Y 1 x Y 304	36 35 T	48.6	17.1	2.9	31.4	45.7	22.9	31.4

Attenuation:-			Lag phase:-			Characteristics of parent strains				
Good	Med	Poor	Short	Med	Long	Y	Flocc	Head	Att	Lag
33.3	0	66.7	0	33.3	66.7	1	III	++	M	M-L
						7	II-III	+++	G	M-L
0	100	0	100	0	0	1	III	++	M	M-L
						8	III	±	M	S
83.3	0	16.7	33.3	50.0	16.7	1	III	++	M	M-L
						9	III	++	P	S
0	100	0	0	0	100	1	III	++	M	M-L
						10	II-III	++	P	L
17.5	22.2	60.3	10.8	41.5	47.7	1	III	++	M	M-L
						26	IV	-	M-G	S
4.7	21.9	73.4	20.0	41.5	38.5	1	III	++	M	M-L
						80	IV	-	P	S-M
22.9	40.0	37.1	34.3	48.6	17.1	1	III	++	M	M-L
						304	II	±	G	M

APPENDIX 1. CONTINUED.

APPENDIX 1

2. Hybrids of brewery strains of Saccharomyces cerevisiae

Hybrids of yeast strains	Number of hybrids	Percentage of hybrids with:- Flocculence type:-				Head formation:-		
		IV	III	II	I	-	±,+.	++, +++, etc.
Y 7 x Y 8	3	33.3	33.3	33.3	0	0	0	100
Y 7 x Y 10	4	0	50.0	25.0	25.0	50.0	0	50.0
Y 7 x Y 26	113	44.2	15.0	36.3	4.4	23.9	42.5	33.6
Y 7 x Y 80	7	42.9	14.3	0	32.9	71.4	0	28.6
Y 7 x Y 304	1	0	0	100	0	0	0	100
Y 8 x Y 9	1	0	0	0	100	100	0	0
Y 8 x Y 10	15 14 T	7.1	35.7	50.0	7.1	21.4	28.6	50.0
Y 8 x Y 304	2	0	100	0	0	0	100	0

Attenuation:-			Lag phase:-			Characteristics of parent strains				
Good	Med	Poor	Short	Med	Long	Y	Flocc	Head	Att	Lag
0	100	0	66.7	33.3	0	7	II-III	+++	G	M-L
						8	III	±	M	S
25.0	50.0	25.0	0	25.0	75.0	7	II-III	+++	G	M-L
						10	II-III	++	P	L
13.3	45.1	41.6	12.4	22.1	65.5	7	II-III	+++	G	M-L
						26	IV	-	M-G	S
14.3	28.6	57.1	28.6	57.1	14.3	7	II-III	+++	G	M-L
						80	IV	-	P	S-M
0	100	0	0	100	0	7	II-III	+++	G	M-L
						304	II	±	G	M
100	0	0	0	100	0	8	III	±	M	S
						9	III	++	P	S
7.1	78.6	14.3	28.6	21.4	50.0	8	III	±	M	S
						10	II-III	++	P	L
0	100	0	0	100	0	8	III	±	M	S
						304	II	±	G	M

APPENDIX 1. CONTINUED.

APPENDIX 1

3. Hybrids of brewery strains of Saccharomyces cerevisiae

Percentage of hybrids with:-

Hybrids of yeast strains	Number of hybrids	Flocculence type:-				Head formation:-		
		IV	III	II	I	-	±,+.	++, +++, etc.
Y 9 x Y 26	55 53 T	11.3	9.4	0	79.3	45.3	41.5	13.2
Y 9 x Y 80	20	0	0	0	100	15.0	55.0	30.0
Y 9 x Y 304	11	0	0	0	100	72.7	18.2	9.1
Y 26 x Y 80	78 76 T	92.1	2.63	2.63	2.63	89.5	7.9	2.6
Y 26 x Y 304	40	57.5	10.0	0	32.5	90.0	2.5	7.5
Y 80 x Y 304	20	40.0	10.0	5.0	45.0	90.0	5.0	5.0

Attenuation:-			Lag phase:-			Characteristics of parent strains				
Good	Med	Poor	Short	Med	Long	Y	Flocc	Head	Att	Lag
56.6	7.6	35.8	41.5	50.9	7.6	9	III	++	P	S
						26	IV	-	M-G	S
70.0	20.0	10.0	55.0	15.0	30.0	9	III	++	P	S
						80	IV	-	P	S-M
72.7	9.1	18.2	27.3	54.5	18.2	9	III	++	P	S
						304	II	±	G	M
9.2	23.7	67.1	25.0	53.9	21.1	26	IV	-	M-G	S
						80	IV	-	P	S-M
15.0	45.0	40.0	35.0	37.5	27.5	26	IV	-	M-G	S
						304	II	±	G	M
10.5	10.5	79.0	50.0	25.0	25.0	80	IV	-	P	S-M
						304	II	±	G	M

APPENDIX 1. CONTINUED

APPENDIX 1

4. Hybrids of brewery strains of Saccharomyces cerevisiae with AB 41 and 43, strains of Saccharomyces carlsbergensis

Hybrids of yeast strains	Number of hybrids	Percentage of hybrids with:- Flocculence type:-				Head formation:-		
		IV	III	II	I	-	±,+.	++, +++, etc.
Y 1 x Y 41	4	0	75.0	25.0	0	50.0	50.0	0
Y 26 x Y 41	20	20.0	65.0	5.0	10.0	65.0	35.0	0
Y 80 x Y 41	9	0	88.9	11.1	0	44.4	55.6	0
Y 1 x Y 43	8	0	25.0	37.5	37.5	25.0	62.5	12.5
Y 7 x Y 43	13 11 T	0	27.3	63.6	9.1	18.2	27.3	54.5
Y 8 x Y 43	47 45 T	4.4	28.9	51.1	15.6	17.8	60.0	22.2
Y 10 x Y 43	27 26 T	0	30.8	61.5	7.7	15.4	30.8	53.8
Y 304 x Y 43	15	0	20.0	80.0	0	6.7	66.7	26.7

Attenuation:-			Lag phase:-			Characteristics of parent strains				
Good	Med	Poor	Short	Med	Long	Y	Flocc	Head	Att	Lag
0	25.0	75.0	0	0	100	1	III	++	M	M-L
						41	II-III	±	M	L
0	20.0	80.0	5.0	5.0	90.0	26	IV	-	M-G	S
						41	II-III	±	M	L
0	0	100	0	11.1	88.9	80	IV	-	P	S-M
						41	II-III	±	M	L
0	62.5	37.5	12.5	62.5	25.0	1	III	++	M	M-L
						43	I	±	M-P	S
36.4	45.5	18.2	18.2	72.7	9.1	7	II-III	+++	G	M-L
						43	I	±	M-P	S
8.9	48.9	42.2	37.8	42.2	20.0	8	III	±	M	S
						43	I	±	M-P	S
23.1	42.3	34.6	38.5	50.0	11.5	10	II-III	++	P	L
						43	I	±	M-P	S
13.3	80.0	6.7	33.3	60.0	6.7	304	II	±	G	M
						43	I	±	M-P	S

APPENDIX 1. CONTINUED

APPENDIX 1

5. Hybrids of brewery strains of Saccharomyces cerevisiae with brewery strains of Saccharomyces carlsbergensis

Hybrids of yeast strains	Number of hybrids	Percentage of hybrids with:- Flocculence type:-				Head formation:-		
		IV	III	II	I	-	±,+.	++, +++, etc.
Y 1 x Y 93	24	0	25.0	12.5	62.5	70.8	16.7	12.5
Y 7 x Y 93	2	0	0	100	0	0	0	100
Y 8 x Y 93	2	0	50.0	50.0	0	0	100	0
Y 10 x Y 93	2	0	50.0	50.0	0	0	100	0
Y 26 x Y 93	86	5.8	52.3	32.6	9.3	62.8	23.3	13.9
Y 80 x Y 93	51	35.3	39.2	11.8	13.7	86.3	13.7	0
Y 1 x Y 140	3	33.3	33.3	0	33.3	100	0	0
Y 26 x Y 140	5	40.0	40.0	0	20.0	80.0	20.0	0
Y 80 x Y 140	4	25.0	75.0	0	0	100	0	0

Attenuation:-			Lag phase:-			Characteristics of parent strains				
Good	Med	Poor	Short	Med	Long	Y	Flocc	Head	Att	Lag
8.3	37.5	54.2	37.5	50.0	12.5	1	III	++	M	M-L
						93	I-II	±	M	L
0	50.0	50.0	50.0	50.0	0	7	II-III	+++	G	M-L
						93	I-II	±	M	L
0	100	0	50.0	50.0	0	8	III	±	M	S
						93	I-II	±	M	L
0	50.0	50.0	0	0	100	10	II-III	++	P	L
						93	I-II	±	M	L
17.4	33.7	48.8	11.6	45.4	43.0	26	IV	-	M-G	S
						93	I-II	±	M	L
17.6	56.9	25.5	7.8	45.1	47.1	80	IV	-	P	S-M
						93	I-II	±	M	L
0	66.7	33.3	33.3	33.3	33.3	1	III	++	M	M-L
						140	II-III	±	M-G	M-L
0	40.0	60.0	20.0	20.0	60.0	26	IV	-	M-G	S
						140	II-III	±	M-G	M-L
0	50.0	50.0	0	25.0	75.0	80	IV	-	P	S-M
						140	II-III	±	M-G	M-L

APPENDIX 1. CONTINUED

APPENDIX 1

6. Hybrids of brewery with bakery or distillery strains of Saccharomyces cerevisiae

Hybrids of yeast strains	Number of hybrids	Percentage of hybrids with:- Flocculence type:-				Head formation:-		
		IV	III	II	I	-	±,+.	++, +++, etc.
Y 1 x Y 32	6 5 T	0	0	100	0	0	100	0
Y 1 x Y 33	1	0	0	100	0	0	100	0
Y 1 x Y 261	2	0	50.0	50.0	0	0	50.0	50.0
Y 7 x Y 32	10	0	0	100	0	10.0	70.0	20.0
Y 7 x Y 33	2	0	50.0	50.0	0	0	50.0	50.0
Y 8 x Y 32	32	0	6.2	93.8	0	3.1	96.9	0
Y 8 x Y 33	5	0	0	60.0	40.0	0	80.0	20.0
Y 8 x Y 261	11	0	45.5	54.5	0	9.1	90.0	0

Attenuation:-			Lag phase:-			Characteristics of parent strains				
Good	Med	Poor	Short	Med	Long	Y	Flocc	Head	Att	Lag
0	60.0	40.0	20.0	80.0	0	1	III	++	M	M-L
						32	I-II	-	M-P	S
0	100	0	0	100	0	1	III	++	M	M-L
						33	I-II	±	G	S-M
50.0	50.0	0	50.0	50.0	0	1	III	++	M	M-L
						261	I	±	G	S
10.0	90.0	0	40.0	60.0	0	7	II-III	+++	G	M-L
						32	I-II	-	M-P	S
50.0	50.0	0	50.0	50.0	0	7	II-III	+++	G	M-L
						33	I-II	±	G	S-M
3.1	96.9	0	25.0	56.3	18.7	8	III	±	M	S
						32	I-II	-	M-P	S
20.0	80.0	0	100	0	0	8	III	±	M	S
						33	I-II	±	G	S-M
27.3	54.5	18.2	18.2	63.6	18.2	8	III	±	M	S
						261	I	±	G	S

APPENDIX 1. CONTINUED

APPENDIX 1

7. Hybrids of brewery with bakery or distillery strains of Saccharomyces cerevisiae

Hybrids of yeast strains	Number of hybrids	Percentage of hybrids with:- Flocculence type:-				Head formation:-		
		IV	III	II	I	-	±,+.	++, +++, etc.
Y 9 x Y 33	6	0	0	0	100	83.3	16.7	0
Y 9 x Y 261	14	0	0	0	100	57.1	21.4	21.4
Y 10 x Y 32	18	0	11.1	88.9	0	11.1	72.2	16.7
Y 10 x Y 33	6	16.7	16.7	50.0	16.7	16.7	33.3	50.0
Y 10 x Y 261	5	0	80.0	20.0	0	0	80.0	20.0
Y 32 x Y 304	16	0	0	87.5	12.5	25.0	75.0	0
Y 33 x Y 304	3	0	0	100	0	0	100	0
Y 261 x Y 304	3	0	0	100	0	0	100	0

Attenuation:-			Lag phase:-			Characteristics of parent strains				
Good	Med	Poor	Short	Med	Long	Y	Flocc	Head	Att	Lag
50.0	50.0	0	0	100	0	9	III	++	P	S
						33	I-II	±	G	S-M
35.7	28.6	35.7	14.3	64.3	21.4	9	III	++	P	S
						261	I	±	G	S
5.6	88.9	5.6	22.2	50.0	27.8	10	II-III	++	P	L
						32	I-II	-	M-P	S
33.3	50.0	16.7	16.7	66.7	16.7	10	II-III	++	P	L
						33	I-II	±	G	S-M
40.0	40.0	20.0	60.0	20.0	20.0	10	II-III	++	P	L
						261	I	±	G	S
0	62.5	37.5	18.8	68.8	12.5	32	I-II	-	M-P	S
						304	II	±	G	M
0	100	0	0	100	0	33	I-II	±	G	S-M
						304	II	±	G	M
0	66.7	33.3	66.7	33.3	0	261	I	±	G	S
						304	II	±	G	M

APPENDIX 1. CONTINUED

APPENDIX 1

8. Hybrids of non-brewery strains of Saccharomyces cerevisiae and Saccharomyces carlsbergensis

Hybrids of yeast strains	Number of hybrids	Percentage of hybrids with:- Flocculence type:- IV	III	II	I	Head formation:- -	±,+.	++, +++, etc.
Y 32 x Y 43	54	0	0	20.4	79.6	9.3	90.7	0
Y 33 x Y 43	4 3 T	0	0	66.7	33.3	0	100	0
Y 261 x Y 43	12	0	0	0	100	8.3	91.7	0
Y 32 x Y 33	7	0	0	28.6	71.4	0	100	0
Y 32 x Y 261	14	0	0	42.9	57.1	7.1	92.9	0

Attenuation:-			Lag phase:-			Characteristics of parent strains				
Good	Med	Poor	Short	Med	Long	Y	Flocc	Head	Att	Lag
5.6	68.5	25.9	48.1	37.0	14.8	32	I-II	-	M-P	S
						43	I	±	P	S
33.3	66.7	0	0	33.3	66.7	33	I-II	±	G	S-M
						43	I	±	P	S
25.0	50.0	25.0	25.0	66.7	8.3	261	I	±	G	S
						43	I	±	P	S
42.9	57.1	0	42.9	42.9	14.2	32	I-II	-	M-P	S
						33	I-II	±	G	S-M
7.1	50.0	42.9	18.6	57.1	14.3	32	I-II	-	M-P	S
						261	I	±	G	S

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Yeast Hybridization

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The yeasts used at present in the brewing industry have arisen from conscious or unconscious selections over a very long time, and we have today a variety of strains particularly suited to top and bottom fermentations of many kinds (11).

In traditional brewing practice, the physical behavior of a yeast in the system used and its activity during both primary and secondary fermentation were principal factors in ensuring consistent product quality. Recent trends in brewing technology (e.g., continuous fermentation, large vessels, centrifuges, the production of filtered beers) and the continuing economic demand for efficient plant utilization have put a different emphasis on the types of yeast required, and it is likely that certain properties—e.g., fermentation rate—will receive greater emphasis in the future.

Three approaches to the improvement of present brewing strains were considered by the authors. The first, continued selection from existing strains, was rejected as being a very long term development with the prospect of probably only limited improvement. The second, the use of mutagens, was also rejected since this could equally introduce deleterious properties as well as improvements so that the yeasts produced could well require extensive examination of their metabolism. The third approach, hybridization, appeared best suited to the production of strains with general improvement in brewing properties because of its successful use elsewhere. Hybridization has been used successfully to produce baking yeasts of high fermentation rate (2), to improve distilling yeasts (12), and also to increase alcohol yields in the fermentation of molasses (13,14). The hybridization of brewing yeasts has been examined by a number of workers (1,6,9), and Windisch et al. (19) have patented a highly attenuative hybrid produced from strains of *Saccharomyces carlsbergensis* and *S. diastaticus*. Hybridization has been successful as a method of producing strains where a need has been clearly stated.

The genetical structure of brewing yeasts can be very complex; many strains are known to be triploid, polyploid, or aneuploid (5); they may not sporulate; they may give sterile spores or only one to three spores per ascus. Fertile spores thus may not be haploid, and we will use the term 'mater' for any product of sporulation or any yeast strain capable of mating directly with another.

Fowell (7) reported that the best hybrids are not necessarily derived from either the parent or mating strains with apparently the most desirable characteristics. An example of the reason for this may be seen in the fermentation of maltose, which is clearly of major importance to the brewer. Up to six genes may be responsible for the fermentation of this sugar (15,20) and their effect is additive, i.e., the more maltose genes a yeast contains the faster it will ferment maltose. The way in which these genes segregate during sporulation and recombine in mating will have a major influence on the fermentation characteristics of a new hybrid. It has also been reported that five genes are responsible for the production of invertase (16), and flocculence in a brewing

yeast is thought to be under the control of three dominant genes (18).

Because of both the complex ploidy of brewing yeasts and of the possible additive effect of large numbers of genes in determining overall fermentation efficiency, we have not made any detailed genetical analyses; rather, we attempted to produce improved hybrids by sporulating and mating directly from yeast strains of accepted fermentation or flavor characteristics.

Method

The procedure for producing hybrid yeasts may be followed in Fig. 1.

Samples of parent yeasts grown on MYGP agar were streaked onto slopes of a pre-sporulation medium containing 2.0% glucose, 0.2% potassium dihydrogen phosphate, 0.2% ammonium sulfate, 0.5% yeast extract, and 1.5% agar [1]. The slopes were incubated for 48 hours at 20°C. and the cultures washed off with 0.2 ml. quarter-strength Ringer's solution. Heavy inocula of the suspensions were streaked onto slopes of a sporulation medium containing 0.1% glucose, 0.18% potassium chloride, 0.25% yeast extract, 0.82% sodium acetate, and 2.0% agar [1]. The slopes were incubated for 48 hours, then examined every 24 hours for spore formation using malachite green/saffranin spore stain.

Sporulating cultures were washed from the slopes with 10 ml. of MYGP broth and incubated in this medium for 18 hours at 25°C. to liberate spores from the asci. Dilutions of these cultures in quarter-strength Ringer's solution containing 1% Tween 80 were plated onto WLN agar (10) and incubated for 72 hours at 25°C. Small or unusual colonies, assumed to arise from spores, were incubated onto sporulation agar. Samples of the parent yeasts were streaked out at the same time, and when these showed sporulation the spore colonies were also examined. Non-sporing cultures were assumed to be derived from spores, and subcultures were stored on MYGP agar for further examination.

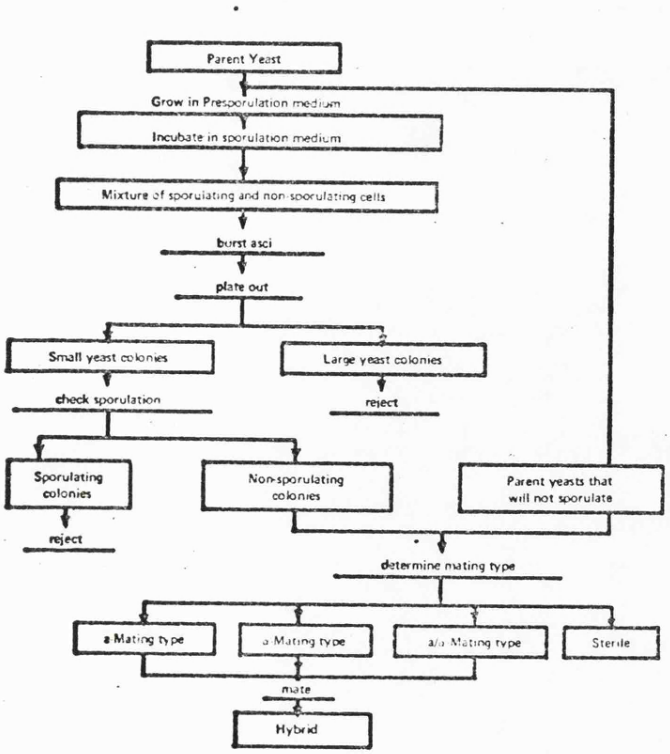

Figure 1. Scheme for producing hybrid yeasts.

[1]G. M. Allen and E. Alm. Private communication to Labatts Brewery, London, Ontario, 1967.

The mating type of these cultures was determined by inoculating on MYGP agar plates with standard a and α marker strains (HR8c and HQ5c, respectively; supplied by R. R. Fowell). Mating was initially detected by zygote formation and confirmed by sporulation of the hybrid on sporulation agar. Hybridization between suitable mating strains was carried out similarly, and hybrids were separated from contaminating 'mater' cells by repeated subculture in 10 ml. of 1.040 SG wort for three successive days at 25°C. During this time the hybrids outgrew the 'maters' and were finally inoculated onto MYGP slopes and stored at 4°C.

Some parent yeasts, which will be discussed later, would not sporulate, but could mate directly with some active mating strains. Hybrids between these yeasts and 'maters' were made by the procedures above.

Genetical stability of hybrids was determined by repeated subculturing in wort and examination of each subculture on WLN agar (10). Flocculence was estimated by a modification of the Chester Method (3). The brewing properties of the large number of hybrids produced were initially examined by recording head formation, sedimentation, and fermentation rate in 10 ml. microfermentations at 25°C. Selected hybrids were compared against their parent strains in 1.25 liter ale fermentations in 900 X 60-mm. diameter tubes at 21°C., and the most interesting hybrids were further evaluated in 20 liter microbrewery ale fermentations at 21°C. In all cases a commercial pale ale brewery wort of SG 1.040 was used for the fermentations.

Discussion

The yeasts chosen for our breeding program showed marked differences in their ability to sporulate. The commercial baking yeasts sporulated well, giving four spored asci. Some brewing strains of *S. cerevisiae* sporulated poorly, usually producing two to three spored asci, and the brewing strains of *S. carlsbergensis* were even more difficult to sporulate, giving two, one, or no spores per ascus.

Table I shows the sporulating ability and mating type distribution of spores produced from the yeasts selected. Only one of the seven brewing strains of *S. carlsbergensis* strains gave any fertile spores. The *S. cerevisiae* strains with the lower sporulating abilities tended to give higher levels of sterile spores. This has been reported to indicate a high ploidy in the parent yeast (5). Mating has been explained by diffusion of a sex factor from the 'α' strain inducing mating activity in the 'a' strain (4), but the high incidence of mater strains in our work with both 'a' and 'α' activity casts doubts on this simple theory. A total of 122 maters were produced, and 55 of these were fertile. Nine hundred thirty crosses were made between these matters, but only 410, i.e., 44% were successful. Crosses in which both parents were brewing strains had a success rate of 15%, but where only one parent was a brewing strain, the success rate was 45%; where both parents were non-brewing strains, the success rate was 80%. These figures illustrate the sterility of mating strains derived from brewing yeasts, and the fertility introduced into yeasts derived from other breeding programs. Three hybrids produced from brewing yeast strains were re-sporulated, and the fertile maters obtained were used to produce 'second generation' hybrids. The crosses between these matters had an 80% success rate, indicating the fertility that may be built into a breeding program once the 'first generation' crosses have been made.

Some yeasts, particularly strains of *S. carlsbergensis* that failed to sporulate, or that sporulated poorly yielding only sterile spores, were found to mate directly with the highly active 'a' and 'α' marker mating strains. These yeasts were

TABLE I
Sporulation of Yeast

Yeast Type	AB Culture No.	Sporulation %	Distribution of Maters a %	α %	a/α %	sterile %
S. cerevisiae, ale-brewing strains	1	6	...	10	10	80
	7	25	25	25	...	50
	8	82	38	38	...	24
	9	2	...	...	...	100
	10	18	45	11	22	22
	80	37	...	...	...	100
	304	53	50	17	...	33
S. carlsbergensis, lager-brewing strains	41	5	...	...	...	100
	90	0	...	...	...	...
	92	10	...	...	...	100
	93	20	...	...	17	83
	94	<1	...	...	...	100
	95	0	...	...	...	...
	140	<1	...	...	...	...
S. carlsbergensis, non-brewing strain	43	50	11	45	22	22
S. cerevisiae, commercial baking strains	32	62	43	14	14	29
	33	31	...	50	...	50
S. cerevisiae, commercial distillery strain	261	36	...	39	...	61

mated with all the appropriate mating strains that had been isolated, but the *S. carlsbergensis* strains would only mate with two of the 55 fertile maters – an 'a' strain from a brewing yeast and an 'α' strain from a commercial baking yeast. These two maters had already been noted for their high mating activity, but we do not know how they derive this. It has been reported that strains of *S. carlsbergensis* are homothallic or homozygous for mating type (5), but this is not necessarily so, since we have found two of our seven strains (AB 93 and AB 94) both 'a' and 'α' in mating activity.

Most hybrids produced were genetically stable, but some broke down to give a number of stable yeasts which could be examined further; others broke down to give a number of products which were themselves unstable, and these were rejected.

A large number of crosses were made between the fertile mating strains of the commercial baking and distilling yeasts and brewing strains, but the presence of the baking and distilling strains appeared to confer non-flocculence to the hybrid. In a further experiment, mating strains from two non-flocculent and fast-fermenting hybrids were mated with maters from three yeasts showing a high level of flocculence. In 153 successful crosses, only 16 hybrids showed any degree of flocculence. Although we have not approached this work from a genetic basis, we have found a repeated absence of flocculence in progeny from flocculent strains, which leads to the conclusion that reports of flocculence being a dominant character (8,17,18) are misleading as a general statement.

The potential improvement in the fermentation rate of hybrids may be seen by examining two hybrids (ABH 10 and ABH 13). Both are 'first generation' crosses of maters from commercial brewing strains of *S. cerevisiae*. In 1.25 liter tube fermentations (Fig. 2), hybrid ABH 10 had a significantly better fermentation rate and attenuation than either of its parents; it had a high head-forming potential and was non-flocculent, giving a high yeast concentration in the beer at the end of fermentation. These parameters were confirmed when compared with another commercial yeast in 20 liter microbrewery fermentations (Fig. 3). Tube fermentations (1.25 liter) of hybrid ABH 13 (Fig. 4) showed that this had a shorter lag phase and higher fermentation rate than either of its parents. Its attenuation was equal to the better parent. It was a non-head former and non-flocculent, leaving a high concentration of yeast in the beer at the end of fermentation. These properties were again confirmed in microbrewery fermentation (Fig. 5).

It is difficult to correlate tube and microbrewery fermentations with actual brewery practice. Fowell[2] has reported that the first hybrids introduced into the baking industry reduced fermentation times by about 50%. It is not thought that the hybrids we have produced would give such large reductions, but time savings of 10 to 20% would appear realistic.

Brewing yeasts have a complex genetic structure and appear to be well selected for their purposes, but this work indicates that hybridization may give a significant

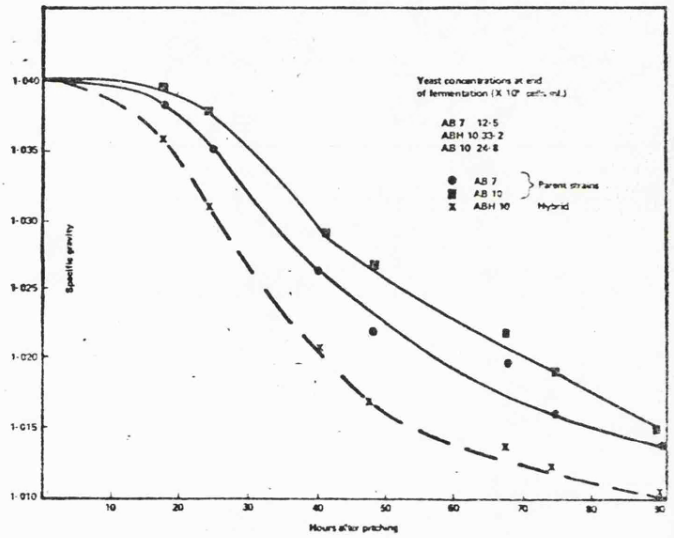

Figure 2. Tube fermentations of hybrid (ABH 10) and parent yeast strains (AB 7 and AB 10).

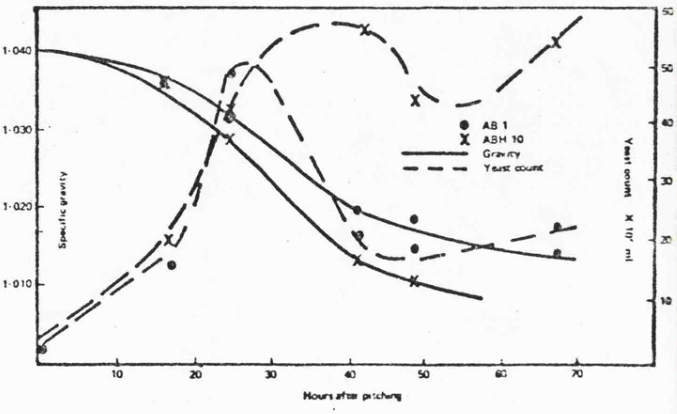

Figure 3. Microbrewery fermentations of hybrid (ABH 10) and commercial yeast strains (AB 1).

[2] R. R. Fowell. Private communication.

improvement in performance if the properties of the required type of yeast are initially well defined.

Summary

Hybridization has been investigated as a possible technique for improving traditional brewery yeast strains, and for introducing properties required by modern brewing technology while retaining characteristic product flavors.

Mass mating techniques were adopted as being particularly suited to the breeding of industrial yeasts, where a general improvement was required by increasing the total level of genetic material rather than the development of an isolated property.

Ale, and particularly lager, yeasts in regular brewery use sporulated poorly and gave few fertile mating strains. In contrast, baking and distilling yeasts of high fermentation rate originally from other breeding programs sporulated well and gave many fertile maters. But in extensive cross mating with brewing strains, they gave few hybrids of interest.

Some hybrids from brewing yeast strains had shorter lag phases and better fermentation rates and attenuating properties than their parents, but lacked flocculence. The hybrids formed sporulated much more readily than did their parents, and gave a high proportion of fertile mating strains for further hybridization.

Hybridization with mating strains from flocculent yeasts generally resulted in non-flocculent hybrids or the loss of other desirable properties. Our data disagree with the simple genetic theory of flocculence advanced by other workers.

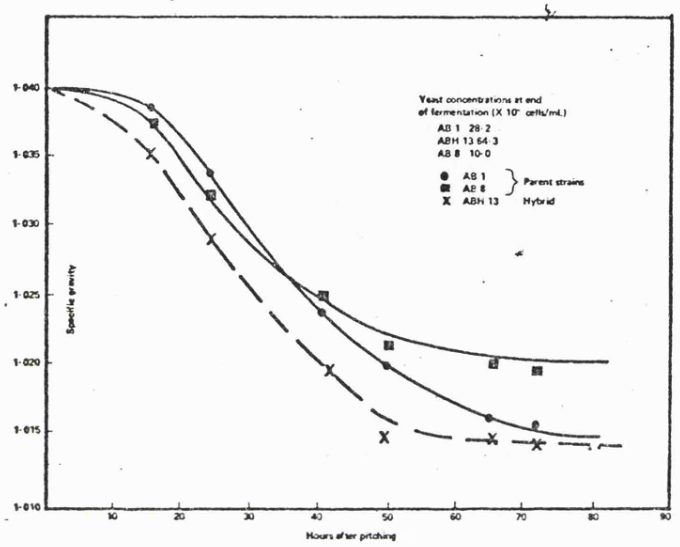

Figure 4. Tube fermentations of hybrid (ABH 13) and parent yeast strains (AB 1 and AB 8).

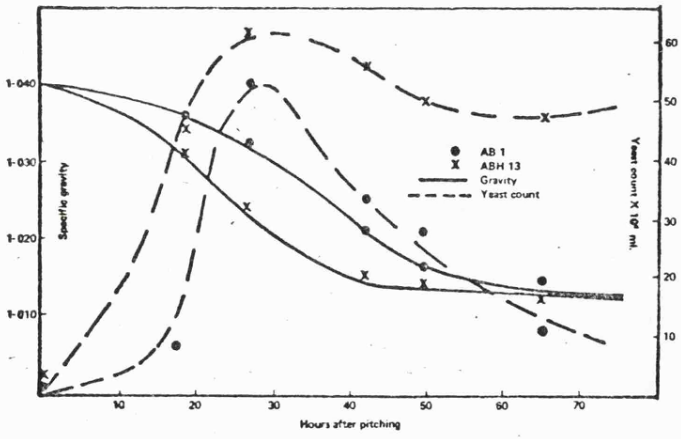

Figure 5. Microbrewery fermentations of hybrid (ABH13) and commercial yeast strains (AB 1).

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